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NEW METHODS FOR THE EXAMINATION OF PIGMENT SYSTEMS *IN VIVO**

F. F. LITVIN

(*Received* 20 *March* 1957)

OPTICAL, and particularly spectrophotometric, methods are becoming increasingly powerful instruments of examination in biology. The importance of these methods for the biologist, striving towards accurate observation of processes occurring in living systems, is determined by their considerable advantages.

(1) The integrity of the organism or of an organ is not disturbed, and it is possible to examine the uninjured organism with physiological functions intact.

(2) It is possible to make exact quantitative examinations of various reactions at the time of their occurrence in the body, of the progressive changes in these reactions and of their relationship to conditions in the external medium.

(3) Because of the fundamental connexion between optical parameters and the properties of molecular structures, spectrophotometric methods permit assessments to be made of minute changes in the state of the molecules of various substances in the body, the degree of their aggregation, and the interconversions of various forms of pigments and other biochemically important compounds with characteristic spectra. In many cases the lability and reversibility of such changes make spectrophotometric methods the only possible methods of examination.

Reports on work in the field of precision, high-sensitivity, high-speed spectrophotometry of biological objects, have recently been appearing in the literature with ever-increasing frequency. The nature and the results of such work indicate that precision spectrophotometry is becoming one of the most penetrating and exact methods for the investigation of biological structures in the living, uninjured state.

A particularly wide field for investigations on the pigment systems of animals and plants in the living state has been opened up. Here the employment of sensitive high-speed spectrophotometry has recently yielded some interesting results.

The development of highly sensitive, rapid spectrophotometric methods has been closely linked with progress in the field of optical, spectral and electrovacuum equipment. The use of photomultipliers, first constructed in this country, has had a very powerful effect. An instrument, combining high sensitivity and freedom from inertia, and permitting continuous objective recording, immediately opened up new possibilities which were unattainable by photographic and visual methods.

One of the first to use the photomultiplier for spectral examinations of biological objects was Lundegardh. He constructed an apparatus for examination of the cytochrome system in living plants, individual organs and tissue homogenates. The apparatus was a combination of a quartz monochromator, a photomultiplier as detector, and a recording microvoltmeter [1] (Fig. 1). The object (roots and leaves

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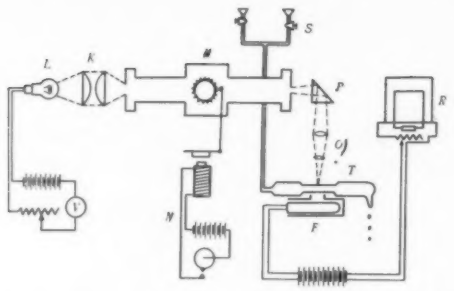

FIG. 1. Apparatus for examination of the absorption spectra of living objects (Lundegardh). *L*—source of light (incandescent lamp); *K*—condenser; *M*—monochromator; *N*—mechanism for rotating the monochromator cylinder; *O*—microscope objective; *P*—prism; *R*—recording microvoltmeter; *S*—system for changing medium; *T*—quartz tube in which the object is placed; *F*—photomultiplier; *V*—voltmeter.

of plants, tissue homogenates, algal suspensions) was placed in a quartz tube connected with an apparatus for changing the fluid medium.

To ensure the maintenance of a normal state of the object and of stable conditions, the time taken for measurement must be reduced to the minimum. With this object in view, the author used an automatic change for the lengths of the waves in the monochromator beam. Turning of the monochromator prism system was effected in "jumps" occurring every 4 sec by means of a specially adapted type of electromagnetic relay. The spectrum recording time did not exceed 15–20 min, and individual lines could be recorded within a few seconds. The combination of such a sensitive detector as the photomultiplier with a quartz monochromator made it possible to attain satisfactory spectral definition, despite the very unfavourable optical qualities of the objects examined (powerful absorption and scatter of light in living tissues). The spectral interval examined did not exceed 0·4–2·0 mμ, and the measurements were carried out over a fairly wide spectral region, from 380 to 640 mμ.

Subsequent improvement of the equipment, while not introducing any fundamental changes, has made for greater accuracy, more completely automatic measurement, and improvement in the conditions for obtaining differential spectra. Particular improvements effected were automatic focusing of the light on the outlet slit of the monochromator synchronously with the turning of the wavelength cylinder and alternating change of the cuvettes containing the specimens (every 4 sec) [2].

In order to examine the rapid and reversible absorption changes during illumination, the apparatus was supplemented by a simple arrangement [3] (Fig. 2): the quartz tube in which the measurements were made was connected to two vessels; after exposure of the suspension in one of them to light, the vessel was raised and the suspension was rapidly displaced along the quartz tube into the other vessel. It was thus possible to observe the absorption changes a few seconds after the exposure to light.

The Lundegardh apparatus, with its relative simplicity, enabled a number of interesting examinations to be made. The power of the photomultiplier to record rapid changes in the light streams was, however, seriously limited by the inertia of the apparatus. Another defect in the method was that the majority of the differential

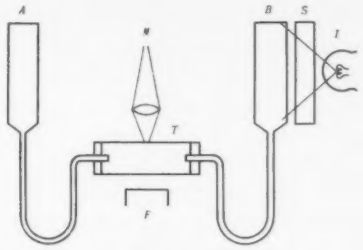

FIG. 2. Arrangement for the observation of change in the absorption spectra of a suspension during exposure to light. *A, B*—vessels containing suspension; *S*—filter; *I*—source of light (acting light); *M*—monochromator; *T*—quartz tube for object; *F*—photomultiplier.

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spectra were obtained one after the other, and not by simultaneous recording; this increased the liability of error in the measurements.

In the last few years reports have appeared on the development of a new apparatus for the examination of rapid and minute absorption changes in living objects. Unfortunately, the apparatus and methods of recording have not been described in the literature, or only very scanty information has been given on them.

In Duysence's work [4] with suspensions of *Chlorella*, reversible absorption changes during exposure to light were investigated. Changes in absorption occurred immediately on exposure to light. The measuring and acting lights were broken down into their spectra. The apparatus was capable of revealing small reversible changes (of the order of 2×10^{-3}) in optical density occurring in an interval of from one to several seconds.

Witt [5], who examined transient absorption changes in the primary processes of photosynthesis, had a still more rapidly acting apparatus at his disposal. He used impulse lighting (argon lamp flashes lasting 10^{-4} sec) and spectrum analysis of the acting and measuring lights. The method used by Witt made it possible to record change in optical density of the order of 10^{-2} and the time relationships of such changes (in intervals of less than 10^{-2} sec). Unfortunately, the author did not dwell on his method of measurement, and did not describe but merely mentioned the "blitzphotometer", constructed for the recording of rapid changes.

Strehler and Lynch [6] made measurements of phototropic absorption changes in *Chlorella* by means of a differential spectrophotometer. The differential spectrum (after exposure of the object to light) was recorded in the course of 30 sec, changes in optical density of the order of 10^{-3} being detected. It appears that these investigators had at their disposal quite a sensitive spectrophotometric apparatus for the measurement of the absorption spectra, but they gave no details whatever of the method and apparatus used for the absorption measurements.

The works of Chance and his co-workers, extending over many years, occupy a particular place in the development of methods for high-sensitivity and high-speed spectrophotometry in biology, and as a result of these, a highly perfected apparatus and method for the examination of pigments and kinetic enzymatic and photochemical reactions actually in living systems (suspensions of bacteria and yeasts, tissue homogenates, etc.) have been developed. To ensure that it would be possible to investigate

both rapid and relatively slow reactions and conversions, the authors set themselves to create a highly stable spectrophotometer, free from "zero drift" (change in the position of the zero) during both slow and rapid signals. This task was successfully accomplished by the creation of an amplifying system (amplifier with direct connexion) and by the elaboration of comparatively simple, but satisfactory, arrangements for stabilization of the light source [7, 8].

As a rule biological objects (cell suspension, tissue homogenates, etc.) absorb and scatter light to an unusual extent. The part of the optical density referable to the component under examination does not usually exceed a fraction of 1 per cent, and the rest goes in scatter and non-specific absorption. As the relative changes in optical density in the course of biochemical reactions and conversions are very small, it was essential that a very high degree of accuracy of measurement should be ensured. As is known, the accuracy of the measurements under optimum conditions with photoelectric recording is limited by intrinsic noises (level of interference) in the measuring and amplifying apparatus. Under such conditions the criterion is the value of the ratio of signal voltage to interference voltage. The greater this ratio, the greater is the accuracy of measurement possible. After the improvements mentioned above, Chance's spectrophotometer was limited in its accuracy only by noises from the spectrophotometer. The efforts of the investigator were therefore directed to a detailed theoretical and experimental investigation of the causes responsible for noise in the separate units of the spectrophotometer (photomultiplier, source of light, amplifier), and in the spectrophotometer as a whole [8], and as a result of this it was possible, by introducing certain improvements, to reduce very considerably the level of noise, so that, in more favourable cases, the ratio of signal voltage to noise voltage reached a value of 5×10^5. Consequently, sources of interference constituted less than 0·00001 of the measured value, and the corresponding error in the measurement of optical density, connected with noises, did not exceed 1×10^{-5}–5×10^{-4} [8]. The task of increasing the sensitivity of the method was thus successfully accomplished, and this made possible the inclusion of an unusually large number of important biological objects and reactions in the field of investigation.

Another important improvement, likewise increasing the accuracy of measurement and being of great value for differential spectrophotometry, was the two-beam method employed by these authors. In this method the beam from the monochromator is split into two so that one of the beams passes through the object and the other through a specimen for comparison. This method is usual in the photographic recording of spectra, but its application when a photomultiplier is being used is more complicated. In his apparatus Chance used modulation (interruption) of the monochromator beam, as a result of which there was interrupted division of the beam (this principle is widely used in installations with photoelectric recording of spectra). As modulator of the light stream, a vibrator, to which was fixed a mirror which served also to switch over the contacts in the amplifying circuit, was used. The principle of its action is shown in Fig. 3. The monochromator beam is alternately (60 times per second) directed to two specimens by the mirror, and falls on the photocathode of the photomultiplier. The impulses from each beam are directed by the switch to different parts of the amplifying circuit; a difference in the optical densities of the specimens determines a difference in the amplitude of the impulses registered by the recording

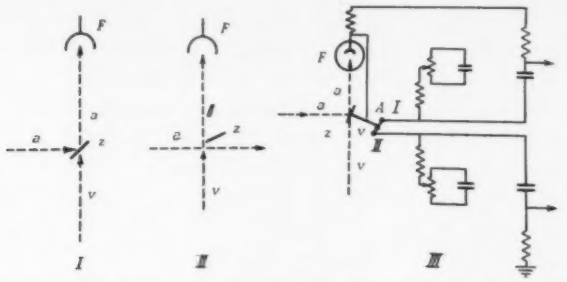

FIG. 3. Principle of the action of the oscillating mirror in the differential two-beam method (Chance *et al.* [8]). *I*—oscillating mirror reflects beam on to the photomultiplier; beam *v* does not reach it; *II*—after 1/120 sec the mirror allows passage of beam *v* to the mirror (beam *a* not now incident); *III*—oscillating mirror serves at the same time to switch contacts, directing the impulses from the photomultiplier (from the two beams of light) to different parts of the amplifier circuit accordingly (mirror in position *I*); *a*—beam passing through object under examination; *v*—beam passing through the comparative specimen; *A*, *B*—contacts; *z*—oscillating mirror; *F*—photomultiplier.

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apparatus. Thus, the measurements are effected differentially—there is direct measurement of the difference in the light passed through the two objects (the object to be examined and the specimen for comparison), and the two samples are compared simultaneously, which accounts for the high degree of mensurational accuracy.

The development of a two-beam arrangement with beam modulation offers a number of important additional advantages. The principle of beam switching makes it possible to use only one photomultiplier, and it is placed directly behind the object being examined. This increases the sensitivity of the apparatus in the examination of biological objects (which usually scatter light considerably), as a considerable part of the scattered light falls on the photocathode; this renders the system for focusing the light on the photocathode superfluous. The impulse arrangement (with modulation of the light stream) creates the most favourable conditions for the operation of the photomultiplier—exhaustion of the photocathode, overheating, etc., are less in evidence. Modulation of the beam can be effected more simply than, and has advantages in the matter of speed over, mechanical alternation of the cuvettes.

Chance, Yang and Legallais [9] constructed highly efficient recording spectrophotometers for biological investigations. In one such spectrophotometer Yang used a Beckman type monochromator apparatus with quartz prisms and an autocollimating arrangement (Fig. 4) [9]. The beam issuing from the monochromator was split by means of an oscillating mirror, and after passing through the specimen fell on

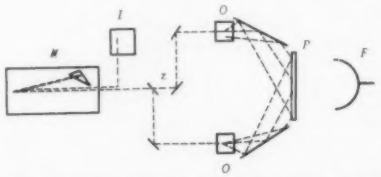

FIG. 4. Two-beam spectrophotometer for biological investigations. *I*—source of light; *M*—monochromator; *z*—oscillating mirror; *O*—specimens; *P*—light-diffusing plate; *F*—photomultiplier.

the photocathode. It is important that the two beams are balanced (i.e. give a null reading in the absence of absorbing substance). Equality in the intensity of the beams is attained by frequent alternation of the beams (oscillating mirror), and an identical geometrical relationship to the photocathode is ensured by a light-scattering plate in front of the photocathode. The arrangement also includes a conversion apparatus for the translation of the indices of the apparatus directly into units of optical density. The results of measurement are automatically recorded in the form of a continuous curve on the linear scale of the wavelengths. In view of the special importance of stabilized amplification automatic amplification control is provided. In many respects this spectrophotometer is similar to that of Chance [8]. Some of the points about these two-beam spectrophotometers are: spectral region 370–600 mμ; spectral interval 3·5–7·5 mμ; error in the measurement of optical density, connected with noise from the multiplier, 10^{-4}–10^{-5}; speed of recording over the spectrum, 6 mμ/sec. For the examination of specimens the parameters of which changed little with time, use was also made of the one-beam method, in which the photocurrent was compensated by a controllable current of opposite direction. The authors developed certain variant apparatuses. Thus, one modification made it possible to measure the absorption by the object for two lengths of waves in relation to time, and this enabled the kinetics of biochemical reactions to be followed by study of the change in the relative concentrations of the two components. Here, the beams from two monochromators, yielding light of two definite wavelengths, were applied alternately to the object under examination. The switch of the beams was effected by the same oscillating mirror [10]. This apparatus was also used to examine the kinetics of reactions; in such cases particular care was taken to ensure that the acting light did not fall on the photocathode as a result of scatter from the object. For this purpose, in addition to crossed light filters, there was in front of the object, in the amplifier circuit, an input capacitance, preventing the incidence of the constantly developing photocurrent (from the acting light); the light to be measured was modulated [11] (Fig. 5). In Chance's work special attention was given to the elaboration of a method and apparatus for the examination of the kinetics of rapid reactions in which respiratory pigments took part. In order to observe the course of these reactions, Chance used a method of current acceleration and arrest which made it possible to detect changes in intervals of from 5 msec to several minutes. The essence of the method lay in the rapid driving of

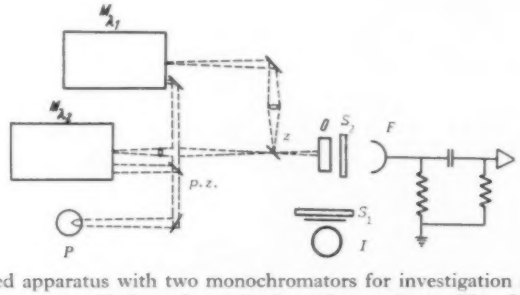

FIG. 5. Modified apparatus with two monochromators for investigation of the effects of photodissociation. *z*—oscillating mirror; *L*—incandescent lamp; $M\lambda_1$, $M\lambda_2$—monochromators; *I*—illuminating lamp (acting light); *O*—object; S_1—yellow light filter; S_2—blue light filter; *F*—photomultiplier; *p.z.*—half-silvered mirror.

the suspension, to which the reagents were added through the photometry tube. The speed of flow in the tube reached 5–10 m/sec. In this way the moment of combination of the reacting mixture with the suspension was fixed accurately by means of an oscillograph. Rapid absorption changes were detected by the photomultiplier and, after amplification, were recorded oscillographically [7, 8].

The works of Chance and his colleagues can be regarded as the greatest achievement in the field for the development of sensitive and high-speed spectrophotometric methods for the examination of biological systems in their natural state. A point which should be particularly noted is that the accuracy and sensitivity of the method makes it possible to extend the range of objects and processes that can be examined. A high degree of stability ensures consistency in the results of measurements; automatic registration and recording of the results in a convenient form reduces the work of measurement considerably.

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Reversible absorption changes in living leaves during their exposure to light were studied by Bell [12]. The apparatus constructed by him was a differential two-beam photometer. The light of an incandescent lamp was divided into two beams; one for measuring (passing through the leaf being examined) and a compensating beam the intensity of which could be varied by means of a diaphragm. Both beams were modulated by a rotating sector disk (4 rev/sec), and then fell on the photomultiplier placed in an Ulbricht "sphere" type vessel (for the collection of scattered light). After amplification by a resonance amplifier the impulses were rectified and fed to the galvanometer. When the intensities of the beams were equal there was no alternating element in the photocurrent, and the galvanometer registered zero. After equalization of the beams by means of the diaphragm, the leaf under examination was exposed to the acting red light. Transmission through the leaf was then changed, an indication of the appearance of an alternate element in the photocurrent of the multiplier (deflexion of the galvanometer needle). The apparatus was capable of revealing quite small changes in the transmission coefficient (to 0·1 per cent) and allowed the course of these changes to be studied in their time relationship. Thus, the combination of a differential two-beam method with light modulation and resonance amplification enabled the author to achieve satisfactory results with a relatively simple apparatus.

Parallel with rapid-absorption spectrophotometry, methods of fluorescence spectrophotometry also began to be developed for the study of pigment systems in the living state.

In this field investigators encountered a number of serious methodological difficulties. The output of luminescent living objects is very small, and therefore the light receivers used must be very sensitive, and the monochromator light must be strong. It must also be reckoned that photomultipliers for the red region of the spectrum (in which pigments such as chlorophyll, protochlorophyll, bacteriochlorophyll and others are fluorescent) are less sensitive than photomultipliers for shorter wave regions. Equally complex is the problem of focusing the light of fluorescence on the photocathode. These difficulties were overcome in the works of French, Virgin and Smith, who examined the spectra of the fluorescence of living photosynthesizing organisms (suspensions of algae and chloroplasts, leaves of plants). In their apparatus [13, 14] the light-exciting fluorescence passed through a powerful monochromator with a diffraction reflecting grating, and was focused on the photocathode

of the photomultiplier. These investigations employed modulation of the light stream: the light of fluorescence was interrupted by a disk rotating 150 times per second, and the photocurrent was fed to an alternating current amplifier. Inequalities at different parts of the spectrum in the sensitivity of the photomultiplier and in the intensity of the beam from the monochromator were automatically corrected. The recording of the spectra of fluorescence was also automatic. The authors emphasized the need to have a powerful source of light and the importance of stability in the working of the apparatus. In this connexion it was found advisable to change to a modulation frequency of 120 c/s, which corresponded to the frequency of modulation of the light source and the electricity supply. It was also found desirable to extend the region in the spectrum for the recording of fluorescence from 750 to 770 mμ [15].

An interesting and promising method was developed by Strehler and his co-workers for the investigation of the fluorescence in relation to photosynthesis [16, 17]; the object used was a suspension of chloroplasts or chlorellae. To increase it's sensitivity the photomultiplier was placed in a thermostatically controlled chamber at the temperature of liquid nitrogen (this reduced sharply the fluctuation of the "dark" current, that is, it reduced the level of noise). The photomultiplier with the corresponding circuit for amplification and impulse take-off functioned as a quantum counter. The suspension under examination circulated through a closed system of tubes, at one point in its course being exposed to light and then passing in front of the window of the photomultiplier. In this way fluorescence was recorded at various intervals after exposure to light (from immediately after up to intervals of 0·1 sec). The apparatus permitted investigation of changes in fluorescence dependent on temperature, inhibitors and other factors, and also in relation to the duration of the dark period. The action spectrum for fluorescence was traced in relative units. The fluorescence was recorded as a whole, but it was also found possible to make certain spectral measurements. It should be noted that the method was a highly sensitive one, as the fluorescence detected was three orders weaker in intensity than luminescence (at an interval of 0·1 sec after exposure to light).

To illustrate the achievements in the spectrophotometry of the pigment systems of living objects, we give a brief review of the results obtained in investigations made with the apparatuses described above.

Lundegardh carried out a number of investigations on the cytochrome system on actual living roots, leaves (of wheat, maize) and suspensions of *Chlorella* [1, 2, 18]. The differential spectra obtained by the author enabled quantitative examinations to be made on the balance between oxidized and reduced forms of cytochromes in living roots, on the effect of various factors (salts, acids, inhibitors) on this balance, on the oxidation–reduction reactions of cytochromes and on the dynamic changes occurring therein. The author demonstrated slight but definite changes in the absorption spectrum of a suspension of *Chlorella* and of leaves after exposure to light, which he associated with photo-oxidation of the cytochrome [3].

Still more comprehensive examinations of the cytochrome system were made by Chance and his co-workers, who employed the perfected technique described above. The sensitivity of the method employed made it possible to include even turbid suspensions of yeast cells and homogenates of muscle tissues and whole muscle in their cycle of investigations. From the differential spectra obtained it was possible

to identify different components in the cytochrome systems of different objects, and to make a quantitative assessment of the absolute and relative concentrations of cytochromes in the cells. The extremely great possibilities of the method were evident in the investigations on the intracellular reactions of cytochromes and pyridine nucleotides. In particular, the kinetics of the oxidation–reduction reaction of cytochrome oxidase in yeast cells were studied at intervals of from 5 to 30 msec [10, 11, 19]. A number of interesting investigations was made on the photosynthetic reactions of purpurin-producing bacteria on exposure to infra-red light, and on the photodissociation of CO_2-cytochromes in yeast cells. Of interest also are the differential absorption spectra of muscle under aerobic and anaerobic conditions.

Methods of sensitive and high-speed spectrophotometry have proved particularly fruitful in the clarification of the important problem of the direct demonstration of the primary products of photosynthesis in living systems under natural conditions and free from injury. This problem could not be approached by the ordinary methods because the concentration of products remained stationary for only very short periods of time, and the products themselves were extremely labile. It is an interesting point that most authors who have investigated absorption changes resulting from exposure to light have demonstrated changes in the interval 515–525 mμ particularly, which is characteristic of the product of the photoreduction of chlorophyll, as discovered and investigated by Krasnovskii and his co-workers.

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Duysence [4] observed reversible changes in the absorption spectra of *Chlorella* during exposure to light. Slight changes in optical density (of the order of $2–3 \times 10^{-3}$) at 515, 420 and 478 mμ, occurring in an interval of from one to several seconds, were demonstrable. The author interpreted the differential spectra obtained as the result of reversible phototropic changes in cytochrome and some other pigment. Similar investigations had been made earlier by the same author with a suspension of bacteria [20].

In experiments to investigate the transient absorption changes in the primary process of photosynthesis, Witt demonstrated diminished absorption at 475 mμ and the appearance of a new band with a maximum at 515 mμ. Relative absorption changes of the order of 10^{-2} disappeared in the course of 2×10^{-2} sec [5].

Recently a paper by Strehler and Lynch has appeared on new maxima in the differential spectra of a suspension of *Chlorella* in addition to those noted by Lundegardh and Duysence (at 648 and 660 mμ) [6]. The authors report interesting findings on the relationship between the sign of the spectral changes and the duration of the exposure to light and of the dark period.

Examining the absorption changes in leaves during exposure to light, Bell observed definite reduction in the transmission coefficient of a leaf in the spectral region around 515 mμ; the magnitude of the effect varied with the frequency and duration of the excitatory light, and saturation was observed with relatively low light intensities. The author considered that these absorption changes can be determined by the formation of a certain quantity of photo-reduced chlorophyll during the exposure of the leaf to the light.

Interesting results were also obtained in investigations of the spectra of fluorescence in living plants. Thus, French and Young [13] examined the spectra of

fluorescence in red algae; the results, in the opinion of the authors, indicated the migration of energy from phycoerythrin to phycocyanin. Virgin [14] examined the fluorescent spectra of chloroplasts and leaves and the effect exerted on them by light-scattering and internal reabsorption. French [13] demonstrated fluorescence maxima in living leaves, which corresponded to chlorophyll *a*, and the absence of chlorophyll *b* and *c* maxima was ascribed by the author to the transfer of energy to chlorophyll *a*.

In the works of Smith [15] the methods of fluorescent spectrophotometry were used for investigation of the rapid conversions of protochlorophyll into chlorophyll, and a number of interesting facts was discovered; the actual occurrence of this process has not, however, been observed directly in the leaf as yet.

The comprehensive experience of the research physicists who have developed sensitive and high-speed methods of spectrophotometry with photomultipliers should prove useful in the elaboration of new equipment for total-absorption and fluorescent spectrophotometry. We have mentioned only some of these works—those relating to investigation on photosynthetic pigments. Livingston and other authors examined the phototropic changes in chlorophyll in solutions. The investigation of the absorption spectra of chlorophyll has been conducted under two different sets of conditions: with constant exposure to a light of relatively low intensity, and with intermittent lighting of high intensity [21, 22].

For the recording of phototropic changes in chlorophyll, a special apparatus was constructed [21, 22] in which the light from the source (incandescent lamp) passed through a collimator, a vessel containing chlorophyll solution, and then through an interference filter to the photomultiplier. The cuvette containing the solution was connected with a Tunberg tube in order to create a vacuum and was placed in a thermostatic water-bath. The interrupted lighting was obtained from three photographic flash-lamps, which gave short and intense flashes (5×10^{-4} sec). After amplification, the signal from the photomultiplier was fed to the γ-plates of the oscillograph, calibrated by means of neutral grey light-filters. The horizontal sweep was switched in a very short time after the flash of the photographic lamps. Any possibility of scattered light being recorded was thus excluded. On replacement of the photographic lamps by ordinary lamps, the apparatus could also be used for the recording of absorption changes during constant illumination. Terenin and Karyakin [23] examined the changes in the absorption spectra of photosynthetic pigments exposed to the action of very intense transient light impulses. A review of works on spectrophotometry with impulse light was given by Karyakin [24].

There are comparatively simple apparatuses embodying a photomultiplier and oscillograph for the investigation of processes arising from light exposure in relation to time [25, 26, 27]. Perfected methods for the investigation of relaxation processes were developed by Tolstoi and Feofilov [28, 29]. The combination of photomultiplier and oscillograph, which determines the high sensitivity and low inertia of the method, could prove very useful for investigations on the kinetics of processes in biological systems (with certain modifications of the method).

Experience in the rational use of the photomultiplier has been accumulated in works on sensitive spectrophotometry in a great variety of different investigational fields, and a number of general techniques and applications has been developed [30]. The principle of the modulation of the light streams has been widely applied. In

addition to the important advantages already mentioned (replacement of the beam by the two-beam method, separation of the acting and measuring lights, etc.) this principle has a whole series of purely technical advantages, increasing sensitivity and stability: (1) modulation makes it possible to employ selective frequency amplification; this reduces in considerable measure the fluctuation spectra and consequently, by lowering the level of noises, increases sensitivity; (2) the amplitude value of the impulses from the light stream can be considerably greater than the intensities of a constant light fed to the photomultiplier, and this simplifies the problem of measurement within wide intensity limits; (3) the problem of compensation for, or reduction of, the constantly present "dark" current is dispensed with and "zero drift" of the apparatus disappears; (4) exhaustion of the photocathode of the photomultiplier is less in evidence.

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Various methods of modulating the light streams by means of toothed disks, oscillating mirrors and so on, have been described in the literature [30]. Automodulation of the source of acting (excitatory) light can be used in investigations on fluorescence.

The amplifying circuit for signals reaching the photomultiplier is very important. Amplifiers for both alternating and constant current are used. For maximum utilization of the principle of modulation, however, alternating current amplification is more advantageous; also, alternating current amplifiers are more stable in operation and offer a number of technical advantages (lower noise level, exclusion of dark current and zero drift, operation independent of the constant anodal resistance, and consequently, of the leakage current of the photomultiplier). Alternating current amplifiers are being increasingly used in spectrophotometric investigations with photomultipliers.

The special requirements in an amplifier of this type are: (1) selective frequency amplification, which ensures sharp contraction of the fluctuation spectrum; (2) a large amplification coefficient; (3) linear amplification within fairly wide limits (as the non-linear amplification of the photomultiplier does not exceed 3 per cent); (4) considerable stability in operation and a low level of interference (the level should not exceed that of the intrinsic noise of the multiplier).

These requirements can be met by any methods of selective amplification. Ordinary resonance amplifiers with circuits consisting of capacitance and inductance have, however, considerable damping decrements; that is why amplifiers with negative feedback and T-form RC filters are often preferred. Such amplifiers can operate at very low frequencies with great selectivity. The circuit of such an amplifier is given in the paper of Kessler and Wolf [31]. In another work [32] an amplifier which embodies two "bridge T" type filters is described. A narrow-band cascade with a T-filter was used by Sobolev and his co-workers [33]. Good results were obtained by Sushchinskii and his co-workers. The amplifier described by them also embodied a T-filter. They detailed the advantages of this type of amplifier over direct current amplifiers. The amplifier described by Sushchinskii [34] possessed satisfactory stability in operation and a low noise level with a high amplification coefficient. Rodionov and Osherovich [35] in their apparatus used an amplifier with negative feedback, as a result of which its stability in operation was increased and chance changes in the modulation frequency were avoided.

So far only the first steps have been taken in the field of sensitive and high-speed spectrophotometry of biological objects; as yet the field of investigations developing these methods is not wide. The results obtained nevertheless testify to their importance and possibilities, primarily because these methods are in accordance with one of the fundamental trends in contemporary biology—the direct study of processes in the living organism.

It should be noted that the apparatus and methods of precision spectrophotometry are still fairly complicated for the research biologist, but satisfactory results can certainly be obtained with even simpler apparatuses.

Constant improvement in the quality of photomultipliers and the perfecting of optical and electronic equipment are constantly extending the possibilities of precision spectrophotometry and expanding the field of its application in biology, rendering it one of the most promising methods for the study of living systems.

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ANALYSIS OF THE TENSION-TIME RELATIONSHIP FOR THE ISOLATED MUSCLE FIBRE*

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IN a study of the tension–time relationship for amphibian muscle, Lucas [1, 2] demonstrated that in a number of instances it was not a straightforward relationship, but consisted of two or three intersecting curves. From a comparison of the tension–time curves obtained with motor nerve and with various parts of normal and curarized muscle, Lucas concluded that these distinct curves corresponded to three excitable elements entering into the composition of the nerve–muscle preparation: the muscle fibres (α-curve), the motor nerve (γ-curve) and the motor endings (β-curve).

Lucas's results were later confirmed by a number of investigations [3–11], but some authors advanced other explanations, differing from that given by Lucas, for the complex composition of the whole muscle tension–time curve. Thus, Lapicque [12, 13] and Moor and Brücke [14] were inclined to regard the α–β–γ discontinuity in the muscle tension–time curve as a peculiar form of artefact, the cause of which lay in the methodological conditions of stimulation. Jinnaka and Azuma [3] showed that in certain cases the tension–time relationship curve for muscle might consist of more than three separate curves. Phenomena similar to the α–β–γ discontinuity in the tension–time curve for muscles have been observed by Umrath [15], Sakomoto [16] and others during stimulation of the nerve trunk, in which the tissue composition of the excitable elements was homogeneous. It is obvious that the explanation given by Lucas for whole muscle is not applicable to either of these cases. For this reason the authors who observed these phenomena regarded them as resulting from the presence in the composition of the muscle or nerve of fibres differing in their excitability. Recently, Praglin [11] advanced the hypothesis that the cause of the complex composition of the tension–time curve lies in the differing conditions under which excitation develops in muscle tissue in relation to very short and more prolonged stimulations.

Lucas's views on the nature of the complex composition of the tension–time curve for whole muscle were subsequently provided with an experimental basis by the works of Rushton [5–9] and Grundfest [10]. These authors showed convincingly that the tension–time curves for muscle and nerve tissue differ materially in their constants, and that, during stimulation of whole muscle, it is possible to obtain combined α–γ curves. It is interesting to note that in the course of these investigations, despite their thoroughness, the β-curve, typifying, according to Lucas, the excitability of the motor terminations, was never once observed.

From the published findings it can be concluded that the explanation for the

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complex composition of the tension–time relationship for whole muscle with the best experimental foundation is that given by Lucas. It should therefore be expected that the tension–time curve for a single isolated muscle fibre should have a discontinuous character, in that it contains all the elements present in whole muscle. This hypothesis is the more appropriate in that for the stimulation of the isolated fibre large fluid electrodes [17–19] are generally used, and these, according to the findings of Rushton [9], afford the best demonstration of discontinuity in the tension–time curve. The aim of the present work was to study the tension–time curve for the isolated muscle fibre, and to analyse it in the light of the findings reported in the literature and mentioned above.

METHOD

Muscle fibres were isolated over their entire length, from tendon to tendon, from the lateral tetanic fasciculi of the ileofibularis muscle of the grass frog. The method used for stimulation of the fibre was that developed by Makarov [20], in which the separation between the two phases, Ringer's solution–air, served as cathode and the anode was placed on one of the tendons of the fibre. In our method, which has been detailed in previous works [21, 22], in order to prevent the upper part of the fibre from drying, the surface of the Ringer's solution was covered by a layer of liquid paraffin, and the cathode was the separation of the phases, Ringer's solution–liquid paraffin.

The tension–time curve of the muscle fibre was determined by means of a condenser chronaximeter with a series of capacities from 450 to 0·002 μF, which corresponded, when a shunt of 100 Ω was interpolated, with durations of the stimulating current of 18 to 0·00008 msec [23]. The threshold was determined visually from the threshold non-graduated contractions. The tension–time curve was generally determined from the longest periods of the stimulating current to the shortest, after which the threshold for the longest stimulating pulse was again measured. An interval of 10–20 sec was observed between the separate stimulations, and the measurement of the entire curve occupied about 10 min. All the tension–time curves were plotted on the logarithmic scale.

RESULTS

In an earlier work we showed [22] that uninjured isolated muscle fibres, kept alive in Ringer's solution, responded during the first 15–20 hr to stimulation of any strength and duration by non-graduated spreading contractions, the amplitude of which did not change materially during this period. Throughout this time there were no significant changes in excitability, so that the tension–time curves obtained in the first hours of the fibre's survival and at the fifteenth to twentieth hours of the experiment are quite close to each other in their constants. A typical tension–time curve for the isolated muscle fibre is shown in Fig. 1(*A*). It will be seen that it is continuous throughout its course and has no obvious angles. From this it can be inferred that the curve obtained reflected the excitability of some one excitable structure, entering into the composition of the nerve–muscle complex. In the method employed, however, the positioning of the electrodes at the middle of the fibre, in the region where the motor nerve enters it, should have facilitated stimulation of the nerve or the motor terminations. It was therefore impossible to determine whether the curve obtained was a "nerve", a "muscle" or a "motor ending" curve. To decide this, comparison was made with the tension–time relationship for muscle fibre and for nerve. The

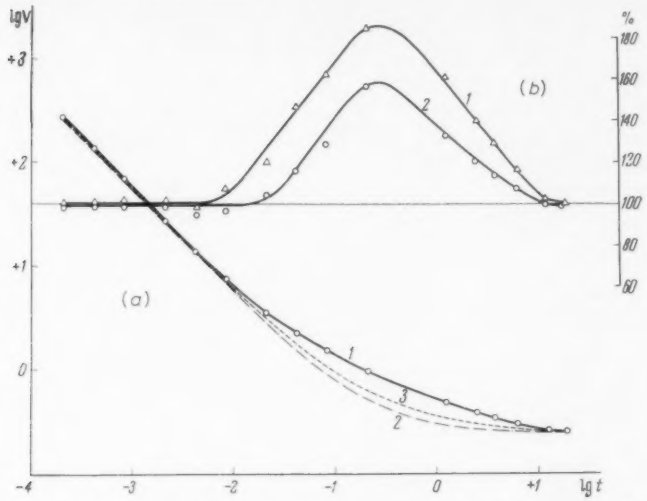

FIG. 1. *A*—Typical logarithmic tension–time curve, obtained during stimulation of a single muscle fibre. 1—experimental thresholds; 2—curve calculated from the formula of Nasonov and Rozental' [23]; 3—curve calculated from the formula of Hill [24]. *B*—Deviation of the experimentally obtained threshold values from the theoretically calculated values. Abscissa: the same as for graph *A*. Ordinate: deviation (theoretical values are taken as 100 per cent). 1—deviation from the Nasonov–Rozental' formula; 2—deviation from the Hill formula.

tension–time curve for nerve was obtained from a nerve–muscle preparation of the sciatic nerve and the ileofibularis muscle. Stimulation of the nerve was effected with simple silver electrodes; the threshold was determined by the first appreciable muscle contraction. A typical tension–time curve for nerve is given in Fig. 2(*A*). Comparison of Fig. 1(*A*) and Fig. 2(*A*) shows that the nerve curve is characterized by an even shorter effective time than the curve for muscle fibre. For purposes of a more detailed analysis of both curves, they were compared with the theoretical tension–time curves, as calculated by the formula of Nasonov and Rozental', $I=(a/t^n)+b$ [23], and by Hill's formula, $E=E_0(RC/k)^{1/[(RC/k)-1]}$ [24]. The corresponding constants were determined from the experimental tension–time curves. The constant n, characterizing in the Nasonov–Rozental' formula the slope of the left rectilinear part of the logarithmic curve, was practically equal to 1 in all cases. In the comparison of the experimentally obtained and theoretically calculated values, the latter were taken as 100 per cent and the results were plotted, with logarithms of the duration of stimulation on the abscissa and the percentage deviation on the ordinate (Figs. 1(*B*) and 2(*B*)). Deviations of the threshold values from the theoretically calculated values of more than ± 10 per cent were accepted as valid. Fig. 1(*A*, *B*) shows that the tension–time curve for the individual muscle fibre is in good agreement with the formula of Nasonov and Rozental' in the regions of fairly short and fairly long stimulation. In the middle region, in the interval from 6 to 0·02 msec, the experimental threshold was higher than might have been expected from the formula. The maximum deviation occurred with stimulation lasting 0·2 msec and averaged (in more than 100 experiments) 75 per cent (64–98 per cent) of the theoretically calculated value.

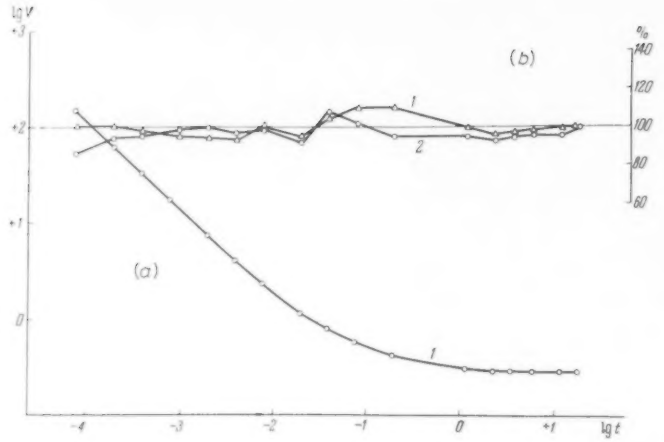

FIG. 2. *A*—Typical logarithmic tension–time curve, obtained during stimulation of nerve. *B*—Deviation of the experimentally obtained threshold values from those calculated theoretically. Notation as for Fig. 1.

Similar deviation occurred when the experimental values were compared with those calculated by Hill's formula. In this case deviation was observed over the interval 3·6–0·04 msec, with the maximum averaging 40 per cent at stimulation durations of 1·2–0·2 msec. The tension–time curves obtained during stimulation of the nerve were in good agreement with the curves calculated by Hill's formula over the whole range of stimulation durations. In most instances there was also similar agreement with the curves calculated from the Nasonov–Rozental' formula (Fig. 2(*B*)). In individual experiments, however, in the interval of stimulation duration, 1·2–0·04 msec, the experimental thresholds proved higher than the theoretical, and the maximum deviation, averaging (in 6 experiments) 25 per cent, lay in the region 0·1 msec.

Thus, comparison of the tension–time relation curves for nerve and the isolated muscle fibre affords evidence that they differ both in the value of the excitability constant and in their configuration. Consequently, the curve obtained from the muscle fibre cannot be regarded as merely the result of stimulation of the terminal fibres of the motor nerve, that is, it is not a pure γ-curve according to the terminology of Lucas. It can, however, be suggested that the deviation of the experimental threshold values for the muscle fibre from the theoretically calculated values is the result of the intersection in this region of tension–time curves for different excitable structures in the nerve–muscle preparation. This hypothesis is the more probable in that in our method the duration of stimulation changed in considerable jumps (6, 1·2, 0·2, 0·08 msec and so on), and this could have masked the α–β–γ discontinuity in the tension–time curve in its classical form. To determine the nature of this deviation of the experimental from the theoretical curve, and consequently also to settle the question of the character of the curve obtained for the muscle fibre, it was essential to exclude completely any possibility of stimulation of remains of the motor nerve and the motor endings. With this aim, experiments were conducted on curarized and denervated muscle fibres.

The curare experiments followed the following plan: in the course of the first 1·5–2 hr the isolated fibre was kept in a chamber filled with normal Ringer's solution, and over this period the tension–time relation curve was determined several times. The solution in the chamber was then replaced by 0·005 per cent curare in Ringer's solution, and change in the excitability of the fibre was studied over a period of 2–3 hr. Control experiments on nerve–muscle preparations (sciatic nerve–ileofibularis muscle) showed that this concentration of curare stopped the transmission of excitation from the nerve to muscle after 10–15 min, while the direct excitability of the muscle was retained without material change for many hours. It will be seen from Fig. 3(*A*) that

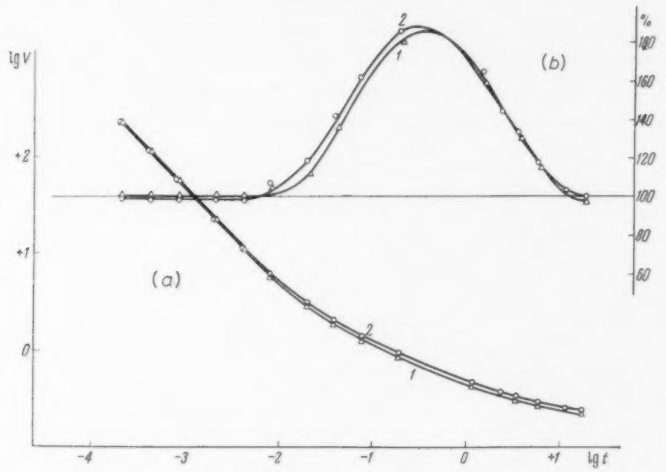

FIG. 3. *A*—Logarithmic tension–time curve for the curarized muscle fibre. 1—for muscle fibre before action of curare; 2—for the same fibre 60 min after beginning of the action of 0·005 per cent curare.

B—Deviation of the experimentally obtained threshold values from the theoretical values calculated by the Nasonov–Rozental' formula. 1—deviation of normal fibre thresholds; 2—deviation of curarized fibre thresholds.

0·005 per cent curare had no real effect on the excitability of the isolated fibre in the period of its activity examined. In none of the eight experiments performed could change in the configuration of the tension–time curve be discovered. The deviation of the ordinary experimental threshold values from those calculated theoretically from the Nasonov–Rozental' and Hill formulae was completely preserved in the case of the curarized fibres (Fig. 3(*B*)). During the period of curare action there were no noticeable changes in the character or extent of the muscular contractions; the responses of the fibre to a single stimulation retained their non-graduated character. Thus, with curare acting in a concentration which rapidly blocked nerve–muscle transmission, the tension–time curve and the character of the contraction of the isolated fibre remained the same as in normal conditions.

For the investigation of the tension–time relation curve in the denervated fibre, the nerve branch from the sciatic trunk to the ileofibularis muscle of one limb of the frog was divided. The frogs so treated were kept for 43–55 days at a temperature of

5°–8° (the experiments were made in January–March), after which a fibre was dissected out from both the denervated and the contralateral muscle. As the nerve branch supplying the ileofibularis muscle is quite short, it was not possible to check the degree of regeneration in the peripheral part of the nerve by stimulation. The state of the motor endings in the denervated muscle were judged by intravital staining with methylene blue (0·025 per cent in Ringer's solution). Whereas in normal muscle methylene blue brings out a large number of motor endings and terminal branchings of the motor nerve, we were generally unable to observe this picture in the denervated muscle. Only in occasional cases was it possible to observe extremely fragmented structures, suggesting by their position the normal motor endings. It should be noted that the period over which we elected to keep the frogs after division of the nerve exceeded the usual periods (15–45 days) employed in the physiological research of other investigators on denervated muscle of amphibians [4, 11, 15]. On this evidence it could be assumed that we were dealing in this series of experiments with fibres whose motor endings had undergone complete degeneration.

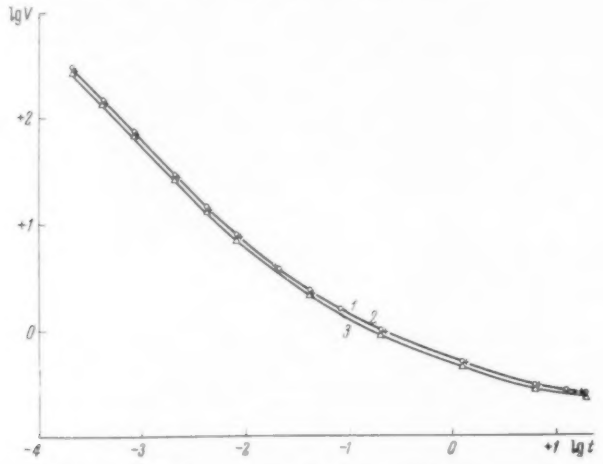

FIG. 4. Logarithmic tension–time curve for denervated and normal muscle fibres. 1, 2—for denervated muscle fibre and fibre from contralateral extremity (each point represents the arithmetic mean of eight experiments); 3—for a normal muscle fibre (each point represents the arithmetic mean of 104 experiments).

Changes in the excitability and the character of the responses were examined in 16 fibres over periods of 10–15 hr: 8 fibres each from denervated and control muscle. Over this period, just as in the experiments with normal muscle fibres, no real changes in excitability were observed, and this made it possible to construct tension–time curves, each point on which was the arithmetic mean of the eight experiments (Fig. 4). Denervated muscle fibres are characterized by exactly the same tension–time relation curve as fibres from the contralateral limb (see Fig. 4). No difference could be discovered in the nature of the responses of denervated and normal fibres.

In this work we were not concerned with the absolute values of the change in excitability in the presence of denervation, and therefore the excitability of the denervated muscle was compared only with the excitability of the contralateral muscle

in the same frog. This may not be a very strict control, as in the case of unilateral denervation functional changes may occur in the contralateral muscle also. Furthermore, the unusually long retention of the experimental animals in captivity may have had an effect on the excitability of the muscles.* The tension–time curve given in Fig. 4, each point on which is the arithmetic mean of 104 experiments on normal muscle fibres, shows that the excitability of denervated fibres is only slightly below normal.

DISCUSSION

The findings arrived at—the smooth character of the tension–time curve of the isolated muscle fibre, its difference from the curve for nerve, the absence of changes in the curve during curarization and in the presence of denervation—indicate that, in the method employed, stimulation of the fibre was always direct, that is, it was the α-curve of Lucas that was being investigated. Thus, in the first place, the deviation of the experimental curve for the fibre from the formula of Nasonov and Rozental' and from that of Hill, noted in the region of stimulations of medium duration, was not, as might have been thought, the masked result of intersection of two or three different tension–time curves. The available data do not permit any determination of the nature of this deviation, but it can apparently be regarded as a characteristic feature of the configuration of the α tension–time curve of a tetanic muscle fibre. Secondly, the presence of motor endings and remnants of the motor nerve, despite conditions conducive to their stimulation (dimensions and situation of the electrodes), was not shown in the tension–time curve obtained from the muscle fibre. It should be noted that the continuous type of curve has been obtained from isolated muscle fibres by the overwhelming majority of investigators [17–19]. The sole exception was the work of Praglin [11], in which the curve for the isolated fibre is described as consisting of two components. Although the curve became continuous under the action of curare, Praglin did not connect its discontinuity with the presence of different excitable structures, but regarded it as the result of conditions associated with the development of the excitation when the muscle was stimulated with currents of different durations.

One of the possible reasons for the absence of the β and γ tension–time curves on stimulation of the muscle fibre may lie in the conditions attaching to its isolation, the nerve fibre supplying it being divided at its actual entrance into the muscle fibre. It is quite probable that, as a result of this, there was rapid functional exclusion of both the terminal branches of the motor nerve and of the motor endings. Ramsey and Street [18] have shown that, whereas immediately after dissection of the fibre the motor endings stain well with methylene blue, this is never seen 24 hr after the isolation of the fibre. Ramsey and Street conducted their experiments at low temperature (4°), and it may be thought that under our conditions (18–20°) the analogous phenomenon developed much more quickly. It may, however, be that this was not a matter of the conditions of preparation, but depended on the fact that motor endings generally do not possess any special electrical excitability differing from that of nerve or muscle. Thus, in Kuffler's experiments on a single fibre with the nerve intact, despite the fact that the stimulating electrode was placed in that region of the fibre

* For the ordinary experiments, frogs which had not been in captivity for more than 10 days were used.

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where motor endings are known to be, a combined α–β curve was never obtained, but only pure nerve or pure muscle curves. Similar results were obtained, as already stated, by the majority of investigators working with whole muscle also. This is possibly the reason why we failed to obtain a β tension–time curve with the isolated muscle fibre. The absence of the γ-curve is more likely to have been determined by division of the nerve branch during the dissection of the nerve fibre.

In conclusion we may dwell briefly on the question of the role of the motor endings in the contractile reaction of muscle tissue. According to Asmussen [26] and Serkov [27], the contraction of the muscle fibre to a single direct electrical stimulation is always graduated. On stimulation of the motor endings or the nerve branch, the contraction acquires a non-graduated character and is subject to the "all or nothing" law. Certain findings reported in the literature [28, 29] and facts arrived at in this present work indicate that exclusion of the motor terminations and the motor nerve (denervation, curarization) has, of itself, no effect on the character of the muscular contractions. Analogous results were obtained in investigation on the action currents of denervated [25] and curarized [30] muscles. All this leads to a belief that the non-graduated contraction is not connected with the functioning of the motor terminations, and may occur with direct stimulation of any point on the fibre. The cause of the non-graduated character of single contractions of the fibre apparently lies in auto-regulation of the magnitude of the excitation spreading along the fibre [22, 31].

SUMMARY

(1) In this work the tension–time curve for a single isolated tetanic muscle fibre of the frog was studied over a wide range of stimulation duration.

(2) The tension–time curves obtained were in good agreement in the regions of fairly short and fairly prolonged stimulations with the theoretical formula of Hill and the formula of Nasonov and Rozental', but in the region of stimulations of medium duration (6–0·02 msec) the actual threshold of the fibre was found to be higher than the theoretically calculated threshold.

(3) The tension–time curve of the muscle fibre differed from that for the nerve both in its constant and in its configuration. It did not change as a result of curarization or denervation of the fibre, and was therefore a purely muscular α-curve.

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ANOMALOUS DIELECTRIC LOSS IN CONTRACTED SMOOTH MUSCLE*

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THE capacity of smooth muscle to undergo prolonged tonic contraction is to some extent related to the physical properties and structure of its macromolecular constituents, which are so disposed that rearrangement on contraction is possible. These properties are related to the polarity of the structural links and to the electrostatic forces between them. The kinetic flexibility is determined by the potential barriers produced by the intermolecular forces. The bonds can be investigated via the frequency-dependence of the dielectric properties [1, 2]. The rate of transition from one statistical equilibrium state (with only thermal agitation present) to another dependent on the external field is determined by the ratio of barrier height to thermal energy. The rearrangement of the polar links involved in this field-induced transition results in relaxation dielectric losses.

Several forms of loss in an alternating field can occur in a structure as complex as muscle [2, 3], namely: (*a*) conduction loss, mainly due to ionic conduction (electrolyte and components of low molecular weight); (*b*) structural loss, related to polarization shifts produced by the field; (*c*) orientation (relaxation) losses, caused by rotational orientation of the dipoles in the field; (*d*) multiphase system losses, caused by absorption currents related to charge flow between microphasic interfaces.

Each form of loss varies in its own way with frequency. Loss of orientation origin produces a region of anomalous absorption near the dipole relaxation frequency, the loss being anomalously high at this frequency, the permittivity falling sharply.

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The frequency at which the tangent of the relaxation loss angle is greatest (tan δ_{max}) is inversely proportional to the dipole relaxation time, i.e. the time in which the orientated state produced by an external field decays to 1/e of its initial value (field off). The relaxation time increases with the opposition to dipole displacement.

One of the dielectric loss maxima for living skeletal muscle lies at 1000–3000 c/s [4, 5], the permittivity also showing dispersion in this range. The anomalous loss region (loss maximum), the shift in the tan δ vs. frequency curve with temperature and the presence of a temperature maximum [5] indicate that we are dealing with relaxation-type loss, which is described qualitatively by the general theory of polar molecules [1, 5]. Studies on the anomalous absorption may throw light on the intermolecular forces and on the packing density of the high-polymer segments in the living material [5, 6].

METHODS

Muscle strips were dissected from the rear adductor of the freshwater mussel (*Anodonta*), the nerve links to the visceral ganglia, comissure and cerebral ganglia being retained or resected at various levels. When the anterior adductor was used the cerebral ganglion was extirpated. The strip thickness varied from 0·8 to 1·2 mm, the strip remaining attached to both valves, the lower valve being held fixed while the upper, separated from the closing ligament, was attached to the myograph lever. The preparation was kept in a moist chamber. The silver electrodes of area 5–6 mm were tightly attached to the two sides of the strip. The experiments in which the temperature was varied were done in a thermostat. Acetylcholine was applied at a concentration of 10^{-3}–10^{-4} as microdrops on the ends of muscle fibres. Check experiments using the same amounts of acetylcholine on strips which had been coagulated by heat, frozen, or treated with formalin, showed that our method was reliable.

The electrical stimuli were applied as 1 sec rectangular pulses of amplitude 2–6 V and repetition time 15–20 sec. When the stimuli were direct the current was limited to 1·2 mA. The properties of the mother-of-pearl and yellow regions were compared by dissecting them out as strips of identical thickness. The changes in tone and the contraction type were judged from the record [7]. To determine tan δ, the leads from the electrodes were taken to one arm of a bridge circuit, a symmetrical bridge being used with standard resistor and capacitor in parallel. The frequency characteristics of the circuit were linear from 500 to 30,000 c/s; the sensitivity was 0·1 per cent. The loss angle was calculated from $\tan \delta = 1/2\pi f RC$, f being the frequency in c/s, R in ohms and C in farads.

RESULTS

The anomalous region was found to lie in the low-frequency range in contracted muscle; Fig. 1 shows the tan δ and capacity curves (full and dashed lines respectively) for posterior adductor muscles from two molluscs. The winter preparation showed maximum loss at 2500 c/s; the tan δ curve is smooth, and the capacitance falls sharply in this region, indicating dispersion in the dielectric constant. The loss maxima ranged from 1250 to 8000 c/s for muscles from 95 preparations, the measurements being made several hours after preparing the specimen, using constant muscle tone. This period was used as the drift found immediately after preparation had then ceased. The same maximum was found on the tan δ curve on repeating the measurements on the same specimen. Since the conductivity as well as the relaxation loss

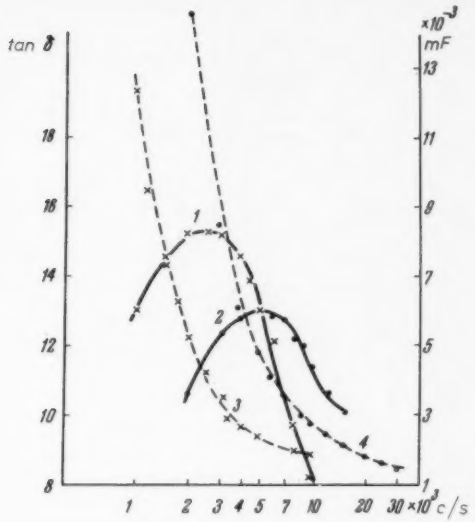

FIG. 1.

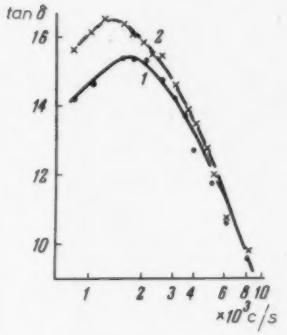

FIG. 2.

FIG. 1. Anomalous electric field energy absorption by a constrictor muscle strip. (1) and (2)—tan δ for different muscle preparations; (3) and (4)—corresponding capacitance variations. Ordinates: tan δ (left) and capacitance (right). Abscissa: field frequency (log scale).

FIG. 2. Low-frequency shift in tan δ produced by electrical stimulation of the muscle. (1)—before stimulation; (2)—after stimulation.

influences the absolute tan δ, the value naturally depends on the strip thickness. It is therefore difficult to compare the absolute tan δ values found in different preparations.

If the temperature was raised within the limits set by contractive or general narcosis in the nervous system, tan δ_{max} moved to the high-frequency side. This agrees with theory, and also with our earlier data on frog muscle [5]. Thus the maximum moved from 1250 to 1750 c/s on raising the temperature from 19 to 32°C. However, adductor muscle is not convenient for temperature variation work as it tends to relax when the temperature is raised.

To study the shift in the maximum on contraction we caused the tone to increase by electrical stimulation to the muscle fibres or nervous system, or by using acetylcholine; relaxation was produced by prolonged rest or by stimulating the cerebral ganglion or extirpating the visceral. Fig. 2 shows the tan δ curve for the winter preparation, the maximum being at 1750 c/s. Electrical stimuli to strips produced increased tone and a stress–strain curve typical of tonic contraction. The maximum was shifted to the low-frequency side in this state, being at 1320 c/s, the resistance here being up by 2 per cent. This rise in resistance, according to previous data [7], indicates tonic contraction. If the tonic contraction was slow, being produced by stimuli to the visceral ganglia or comissure (if the links to both ganglia were retained or resected) the shift was found to be towards low frequencies in 32 cases (see Table 1). In 5 cases there was no shift, and 2 were exceptional (high-frequency shift); these exceptions may possibly be due to contractions of mixed type.

TABLE 1. ELECTRICAL PARAMETER CHANGES IN MUSCLE STRIPS ON TONIC CONTRACTION AND RELAXATION

Date	Action or stimulant	Reaction	Shift in frequency of maximum loss* $\Delta f/f$, %	Resistance change $\Delta R/R$, %	Change in height of maximum $\frac{\Delta \tan \delta, \%}{\tan \delta}$
1955					
1 March	Electrical to muscle layer	Increase in tone	−7·5	+1·7	+1·0
			−31·4	+1·2	+5·2
3 March	,, ,,	,, ,,	−18·2	+4·9	−1·2
10 March	,, ,,	,, ,,	−7·2	+0·5	−2·3
24 August	,, ,,	,, ,,	−16·9	+1·2	+6·2
25 August	,, ,,	,, ,,	−18·7	+1·35	+2·16
1954					
9 August	Electrical to comissure	,, ,,	−13·4	+9·9	0
30 July	Electrical to visceral ganglion	,, ,,			
1955					
21 February	Acetylcholine 10^{-4}	,, ,,	−15·0	+6·5	+1·6
28 February	,, ,,	,, ,,	−15·8	+4·0	+0·5
3 March	,, ,,	,, ,,	−16·6	+3·0	−1·8
2 March	,, ,,	,, ,,	−27·3	+8·0	+2·0
10 March	,, ,,	,, ,,	−49·0	+11·4	+0·6
1956					
9 July	,, ,,	,, ,,	−28·6	+4·3	+3·6
25 July	,, ,,	,, ,,	−36·5	+3·3	+2·0
1954					
2 March	Extirpation of ganglia	Relaxation	+5·0	0	+2·0
8 March	,, ,,	,, ,,	+16·9	−3·87	−2·4
17 July	,, ,,	,, ,,	+11·1	−6·9	+4·4
23 July	,, ,,	,, ,,	+43·0	−15·7	0
31 July	,, ,,	,, ,,	+22·8	−5·37	+3·6
6 August	,, ,,	,, ,,	+24·4	−6·85	−1·59
1956					
4 August	,, ,,	,, ,,	+47·5	+10·2	−13·0

*− denotes a shift toward low frequencies, + one toward high. Increases in R and tan δ are denoted by +.

When the strips lost their tone the maximum loss frequency shifted to the high side. Fig. 3 shows a case where the relaxation was brought about by prolonged rest at 4°C. Curve 1 was taken 2 hr after preparation of the specimen (maximum at 3000 c/s): after seven days (curve 2) the maximum had moved to 5500 c/s; meanwhile the tone had decreased considerably, though the specimen was still excitable. When the excitability had been lost there was a marked shift (to 10,000 c/s—curve 3), the absolute value of tan δ_{max} increasing. Further storage caused the structure to disintegrate and the maximum moved out of the working range (curve 4). The regularity previously found for frog muscle [4, 5], i.e. shift of the maximum to higher frequencies on storage, also occurs with smooth mollusc muscle.

In another series of experiments, relaxation of posterior adductor muscle was brought about by extirpating the visceral ganglia (Fig. 4), the maximum then shifting from 4500 to 5000 c/s. Seven such experiments gave the same result (see Table 1). Relaxation produced by stimulating the cerebral ganglion electrically also caused a high-frequency shift, with a slight change in the absolute value of tan δ_{max}.

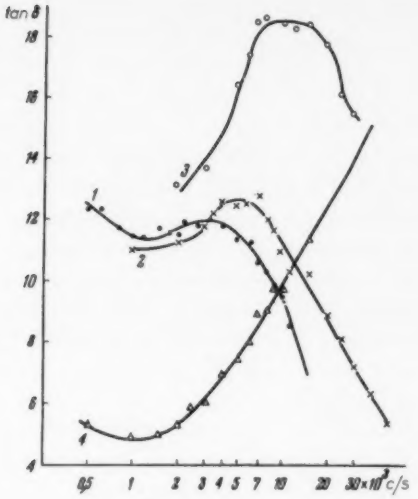

FIG. 3

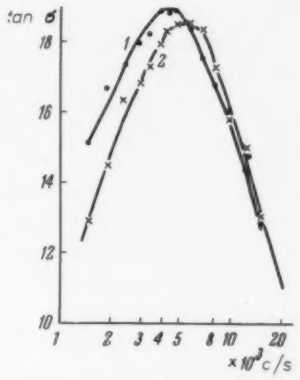

FIG. 4

FIG. 3. Shift of anomalous absorption to the high-frequency side on prolonged use of preparation.

FIG. 4. Shift of tan δ_{max} to the high-frequency side caused by extirpating the visceral ganglion. (1)—tonic contraction state; (2)—partial release.

We used chemical stimulation as well as electrical to see whether the characteristic structural changes accompanying increase in tone were independent of the stimulus. Fig. 5 shows that there is a shift to lower frequencies after applying acetylcholine (3380–2630 c/s), i.e. as for electrical stimulation. The same effect was found in 26 experiments on posterior adductor and in 7 on anterior; in 5 there was no change, and in 2 the shift was to higher frequencies.

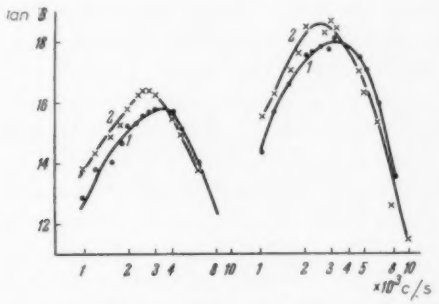

FIG. 5. Increase in tone produced by acetyecholine in two different instances. (1)—before application; (2) after application.

The shift to lower frequencies is seen particularly clearly in the mother-of-pearl layer of the posterior adductor which [8] has more clearly expressed tonic properties. But no clear difference between this layer and the yellow is seen, as regards the position of tan δ_{max}. The nacreous layer was always of much lower resistance (e.g. 25 per cent

down at 2500 c/s), which is in agreement with the general tendency for the more tonic fibres to have the lower resistances at low frequencies, since smaller electrical charges are located at their phase boundaries [9].

DISCUSSION

The data show that muscle tissue can be considered as a polar system with a relaxation mechanism of loss. The anomalous loss and dielectric constant dispersion are found at low frequencies, i.e. in the same range as for some other macromolecular structures, e.g. plastics [10]. Another mechanism (the Maxwell–Wagner), which also results in anomalous absorption, may be caused by structural non-uniformities such as foreign inclusions (droplets in plastics). We shall only consider the relaxation mechanism caused by rotation of polar links, and their overcoming potential barriers resulting from long-range forces.

The relaxation loss in a polymer system is determined by the degree of free internal rotation about single bonds as well as by the sizes of the polar monomer links. The forces hindering rotation are electrostatic in nature. They determine the molecular interaction energy, which is much smaller (at 0·1 kcal/mole) than bond energies. This means that the internal lability of a polar link and its tendency to overcome the potential barrier depend strongly on temperature. The loss maximum shows about a 30 per cent change in frequency for a temperature change of 10°C. We are clearly dealing with changes in some part of the intermolecular forces which are sufficient to break up the long-range order. The shift to high frequencies on storage indicates that the molecular association breaks down.

The experimental curves of tan δ vs. frequency are smoother than the general theory would indicate; this is because the macroscopic properties are the means over enormous numbers of individual molecular phenomena. The molecular packing in muscle is less dense than in a solid, while the dipoles are immersed in what is chemically a liquid, but are also themselves chemically bound into long chains and cannot therefore be greatly displaced. The bonds in such a system would be expected to show a large variety of configurations, so that the relaxation times of the individual segments would be expected to show a continuous distribution, as is indicated by the flat maximum in the anomalous absorption curve. Theoretical calculations on a model of a polar polymer in a non-polar solvent [11] show that the relaxation times give rise to a most probable value of tan δ_{max} which is proportional to the degree of polymerization.

The loss maximum moves to low frequencies when direct electrical stimulation of the muscle or the nervous system, or the use of acetylcholine, causes tonic contraction; the absolute value of tan δ_{max} is then frequently somewhat increased. This latter depends to a considerable extent on the ionic conductivity, increasing with the conductivity loss. Experiments with plastics [12] also show than tan δ_{max} increases with the concentration of polar groups. The rise in tan δ_{max} in tonic contraction is not due to increased ionic conductivity, because the resistance component actually increases, so the rise may possibly be due to an increase in the polar groups. On the other hand, the shift to low frequencies indicates closer molecular packing and strengthening of the bonds between molecules.

We may suppose that some atomic groupings (masking groups) are removed (or

bonded) in tonic contraction, these being groups which prevent the chains coming closer together when the muscle is at rest. These groups then become ineffective, the number of polar groups increases and the chains come closer together. This appears as an increase in the absolute value of tan δ_{max} and a shift to lower frequencies. When the muscle relaxes (this probably being an active process—it is mediated by the nervous system) the blocking groups reappear and displace the chains again, which allows the dipoles considerably more freedom of orientation. This rough picture is in agreement with the idea that removal of tone is not a passive process [13]. The inhibiting action in cross-striated crab's claw muscles are of interest here [14]. The inhibiting nervous impulse can cause reduced tone, possibly by facilitating the freeing of those blocking groups proposed above, and thereby disrupting the aggregated macromolecular complexes which formed earlier during slow contraction, by producing colloid displacements in the reverse order. It is interesting that this drop in tone is not accompanied by an action potential appearing on the muscle membrane [15]. No membrane mechanism seems to be involved in relaxation following slow contraction. It is probable that in our scheme above molecular readjustments on the membranes are not the leading ones.

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The mechanism suggested relates only to tonic contractions, structural processes of quite a different type being at the root of the fast type of contraction (phasic) in mussel adductor muscle [7].

CONCLUSIONS AND SUMMARY

(1) Dielectric studies on mussel (*Anodonta*) adductor muscle have shown that at low frequencies (200–2000 c/s) there is a region of anomalous absorption and dispersion in the dielectric constant. The frequency maximum loss was found to depend on temperature; this indicates that muscle tissue can be considered as a polar system with a relaxation mechanism which can be qualitatively described by Debye's general theory.

(2) Tonic contractions of the above muscle produced by various agents (electrical stimuli to muscle or nervous system, acetylcholine) caused a regular shift in the loss maximum towards lower frequencies. If tone was lost by nervous action the maximum moved towards higher frequencies.

(3) It is supposed that when the tone increases the bonds between molecules, which are electrostatic in nature, become stronger, and that the molecular packing becomes denser. If muscle relaxation is an active process we would expect that the groups which block the polar groups would be activated, and hence (because the bonds are weakened) the molecular chains would be separated.

Translated by J. E. S. BRADLEY

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THE PROBLEM OF METHODS OF RECORDING THE LOCAL ELECTRICAL REACTIONS OF NERVES*

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STUDY of the electrical reaction of a nerve at the actual site of excitation is attended by great difficulties associated with the presence of a so-called electrotonic potential, i.e. the electrical polarization of a nerve caused by the passage through it of a current. The degree of polarization depends on the density of the current lines of force; it is at a maximum beneath an electrode and decreases with distance from that electrode. Thus on stimulation by a rectangular stimulus in the extrapolar region of a nerve, a potential gradient is set up with its maximum at the stimulating electrode. If we record the electrotonic potential at any two points in this region, it can be shown that the potential contains two components: fast and slow. The fast component is the result of the simple ohmic drop in voltage when a current is passed through the fluid and connective tissue sheath of a nerve; it retains the form of the applied potential and exists also in dead nerve; i.e. it is a manifestation of the purely physical properties of a nerve. The slow component, which has the polarization of the nerve as its origin, reaches its steady value exponentially; also it falls exponentially when the flow of current ceases. It originates simultaneously at different points in a nerve, but its rate of rise decreases with increasing distance from the electrode and its steady value decreases in the same way. In a dead nerve the slow component disappears and the simple ohmic current distribution remains.

The electronic potential is recorded together with the action potential of a nerve and does not permit determination of the latter in unadulterated form. This applies particularly to study of the sub-threshold localized nerve response, the magnitude of which does not exceed 1 mV in a normal frog's nerve [1] and which can, in consequence, easily be masked by a higher electrotonic potential. It is not surprising therefore that for a long time a number of authors [2, 3] left the existence of the sub-threshold localized response in the nerves of vertebrates open to doubt.

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This difficulty was overcome in Hodgkin's work [4], in which the existence of a sub-threshold localized response was first proved, by the use of a clever process for deducting the value of the anodic polarization potential from the total potential recorded at the cathode. This method made it possible to reveal a sub-threshold localized response from a single crab fibre. The use of Hodgkin's method to study the nerves of vertebrates gave no positive results. This may be explained by the fact that in a whole nerve trunk the pure sub-threshold localized responses only arise in practice in a small number of hyperexcitable fibres, while they are partially shunted by the mass of the remaining fibres and connective tissue, and the high electrotonic potential of all the fibres is superimposed upon them. This disadvantageous correlation between the electrotonic potential and the local response of a whole nerve trunk does not permit us to differentiate between them by simple graphical subtraction.

In the work by Katz [1] the siting arrangement for the stimulating and recording electrodes shown in Fig. 1 was used; this made it possible partially to neutralize the passive polarization effects and to obtain a correlation between the electrotonic potential and the local response such as to permit the use of a graphical subtraction method.

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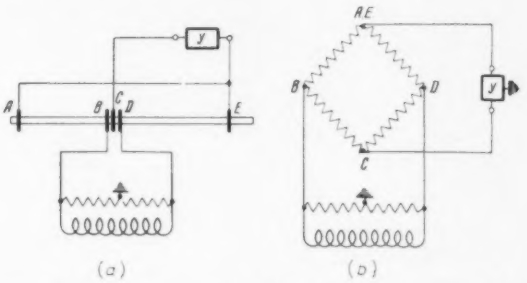

FIG. 1. Stimulating and recording electrode siting arrangements used in Katz's work [1]. *a*—siting of electrodes on the nerve; *b*—the equivalent bridge circuit. This is explained in the text. On both diagrams *y* is the amplifier.

The principle on which Katz's circuit worked was as follows: it is possible, by moving the recording electrode *C* between the stimulating electrodes (Fig. 1(a)), to find the point on the nerve at which the slow component of the electrotonic potential is nil, while a second recording electrode is placed at a point on the nerve possessing an identical reversed potential; in consequence it, too, registers zero potential. In other words the circuit works on the bridge principle, the arms of the bridge being sections of the nerve (Fig. 1(b)). The fast component of the electrotonic potential is compensated by connecting a resistance, with earthed centre point, in parallel with the transformer.

When sub-threshold stimulation is provided by long-duration current pulses (4 μsec) and the electrode *C* is located at the point of maximum neutralization, a smooth line is observed on the oscillograph screen with spikes at the beginning and end of the stimulating pulse. The nature of these spikes is complex. In the first place they are the result of the fact that complete neutralization of the slow component of the electrotonic potential is impossible, compensation being made partly at the expense of the square component. In the second place, additional spikes, the

result of the passage through the circuit of a current caused by the capacity to ground of the transformer, take part in the formation of these spikes. In the case where a transformer of low capacity to ground is used, these spikes are of minimum size; but when the stimulation is increased and a localized response appears they may make observation considerably more difficult.

The following should also be noted as disadvantages of the circuit:

(1) Since we know that a nerve impulse may be propagated from the cathode, incremented or decremented depending on the degree of stimulation [4, 5], recording away from the site of stimulation will, although close to this site, present additional difficulties to the quantitative investigation of the localized response.

(2) The localized response may be partially shunted by the low resistance stimulating circuit. Since this shunting depends on the space distribution of potential, it must differ with changing conditions, and is certain to have a particularly telling effect on the extremely low sub-threshold localized responses.

The conclusion to be drawn from all that I have stated is that the circuit which enabled Katz to be the first to prove the presence of a sub-threshold localized response in the nerves of vertebrates, and thereby to indicate the possibility of a gradual reaction in the nerve pulp, cannot be considered completely successful as regards the quantitative investigation of local responses and their dissemination along the nerves.

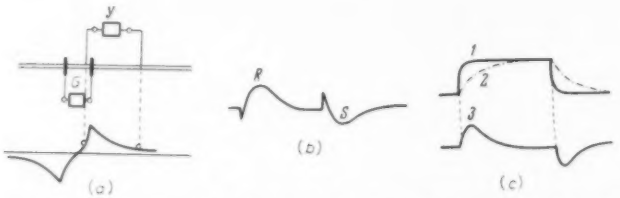

FIG. 2. Stimulating and recording electrode siting arrangements used in the work of Rosenblueth and Ramos [6]. *a*—diagram for the siting of the recording electrodes at isopotential points on a nerve; *b*—oscillograph recording from the work by Rosenblueth and Ramos; *c* 1—electrotonic potential close to the electrode; 2—electrotonic potential at a distance from the stimulating electrode; 3—the difference between them. *y*—amplifier; *G*—generator.

In 1952, Rosenblueth and Ramos [6], working on the spinal cord radicles of cats, proposed a new circuit for compensating the electrical loop. This circuit was based, as was the previous one, on finding isopotential points on the nerve. One of the recording electrodes is located in the interpolar, the other in the extrapolar region at points on the nerve at equal potential. Fig. 2(*a*) shows the distribution of electrotonic potential along the nerve and the arrangement of the electrodes. With sub-threshold stimulation by means of long current pulses (10–20 μsec) an almost smooth line is observed on the screen at the point of optimum neutralization, with spikes at start and finish; the cause of these spikes is the same as in the Katz circuit, i.e. they are the consequence of incomplete compensation of the slow polarization. The response which appears on increasing the stimulation is located on the smooth line between these spikes and can be recorded without subtracting the anodic polarization. Fig. 2(*b*) is an oscillograph recording obtained by Rosenblueth and Ramos for a stimulation

just short of threshold using long current pulses. Two deflexions can be seen on the recording: a negative deflexion (*R*) starting during stimulation and a positive deflexion (*S*) after the end of stimulation. Rosenblueth considered that the first deflexion was the local response, the second a "positive oscillation" of the fibre, of a physiological nature.

This at first glance successful solution to the problem of compensation does, however, give rise to objections. The recording electrodes are located at different distances from the stimulating electrode: one is 0·2–0·5 mm from the cathode, the other 10–15 mm from it. The rate of increase of the electrotonic potential close to the stimulating electrode and at a distance from it are different. In the first case it is considerably higher than in the second [7]. This difference between rates will be registered at the recording electrode notwithstanding the fact that the steady potential value may be the same. In this case the picture on the screen (see Fig. 2(*b*)) will be similar to Rosenblueth's oscillograph recording. Taking into account the fact that the "positive oscillation" is always less than the localized response, one might think that both the true localized response and the residue of the electrotonic potential took part in forming the local response, while the "positive oscillation" is a purely passive phenomenon of this type. In addition, one of the most material objections to Katz's circuit, that it involves recording at a point other than that of stimulation, is effective here also.

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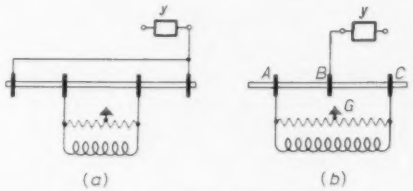

FIG. 3. Stimulating and recording electrode siting arrangements used in the present work. These are explained in the text.

At the beginning of my work I attempted to differentiate the localized response from the electrotonic potential, altering the circuits previously used by other research scientists but retaining unchanged the principle itself on which they worked—the location of the electrodes at isopotential points on the nerve. These alterations led to combining the stimulating and recording electrodes; this made it possible to record the nerve response at the point of stimulation, and to avoid the main shortcoming of the research methods described above. I will describe two circuits which I tested, along with those of Katz, and Rosenblueth and Ramos, during my work.

One of these circuits (Fig. 3(*a*)) differs from Katz's circuit in that the recording electrode *C* is missing, while the stimulating electrodes, connected through the earthed point of the potentiometer to the amplifier earth, are at the same time one of the recording electrodes. The electrotonic potential is compensated, for subthreshold stimulation, owing to the fact that each of the recording electrodes taps zero potential: one recording electrode, being simultaneously both cathode and anode, records the maximum values of reversed polarization; the other is located at points on the nerve where the reversed potentials are identical.

The square component of polarization is compensated by movement of the earth point of the potentiometer. Completely symmetrical siting of the electrodes ensures that the slow component of polarization is entirely and satisfactorily compensated. The spikes left on the screen at the beginning and end of stimulation are for the most part governed by stray capacitances, notably the presence of the capacity to ground of the transformer. Where stimulation almost reaches threshold, a picture of the local response registered at the cathode can be seen on the oscillograph screen. When stimulation is increased to above the threshold, a bulge appears on the right; this bulge is the action potential registered at the anode. In order to avoid recording the action potential, the nerve beneath the anode is killed. The advantage of this circuit over that of Katz is that here recording takes place at the stimulation point, i.e. the genuine localized response is recorded. The quality of compensation is the same as in the other bridge circuits. The shortcoming is the shunting of the recorded potential by the stimulation circuit; owing to the greater distance between electrodes, this shunting reaches higher values in the case in point than in Katz's circuit.

The second circuit (Fig. 3(*b*)) differs from Katz's circuit in not having a split recording electrode located at the ends of the nerve. Here the stimulating electrode are also the recording electrodes, since they are connected with the amplifier earth. Compensation is accomplished owing to the fact that electrode *B* is located at the point of minimum polarization, and the second recording electrode, being at the same time a stimulating electrode, provides zero potential. The square polarization component is neutralized by the bridge, since the potentials at points *B* and *G* are equal. The slow polarization is not fully compensated. Its remnants, compensated by the square current, provide spikes on the screen. Where the stimulation is supra-threshold the recording of the nerve response is triphasic. The first phase is the localized response registered at the cathode, the second and third are the action potential registered at the central recording electrode and at the anode.

The shortcoming of this circuit is that the localized response recorded is in very diminished form, firstly because it is recorded from half the circuit and is thus halved, and secondly because it is shunted by the low resistance of the circuit external to it. The localized response is, in consequence, more than halved on recording. The same is the case with recordings of the anode action potential, i.e. when recording the third phase. When recording the second phase this is not the case, since here there is no subdivision or shunting. As a result an unfavourable correlation between the first and second phases is obtained, and this cannot be altered by killing the nerve beneath the electrode, since the point of compensation may move during the experiment.

Summing up, certain conclusions may be drawn concerning the procedure for recording the localized response using bridge circuits.

It is comparatively easy to arrange for recording at the site of stimulation, but rather difficult to avoid the basic shortcoming of bridge circuits—unsatisfactory compensation. Even attempts to allow for the reactive resistance of nerves by introducing an additional capacitance into the circuit [8] does not provide a better result. Meanwhile the presence of remnants of the slow polarization makes quantitative measurement of the localized response more difficult. Besides this, the unavoidable necessity of using symmetrical stimulation brings with it the phenomenon of shunting of the localized response by the stimulating circuit.

Taking all these circumstances into account, we can arrive at the basic requirements made of procedures for recording local responses:

(1) recording must be performed at the site of stimulation; in short, the cathode must at the same time be the recording electrode;
(2) the electrotonic potential must be satisfactorily compensated, permitting direct observation of the local response without distortion;
(3) the stimulation circuit must not shunt the recorded potential; for this purpose a current generator must be used as the source of stimulation.

Bearing these requirements in mind, I worked out a circuit founded on completely different principles.

I obtained fully satisfactory compensation by feeding a compensating voltage, of the same form as the electrotonic potential, into one of the inputs of a differential amplifier. The generator of the compensating voltage is a summing amplifier; a voltage proportional to the stimulating current is fed into its inputs through several *RC* circuits, independent of each other and with adjustable time constants. The form of the voltage at the output side of each *RC* circuit is determined by its time constant. A voltage is produced at the output side of the summing amplifier equal to the sum of the input voltages. A voltage of the required form may be produced at the output side by altering the time constants of the *RC* circuits and the amplitudes of the individual components. A diagram showing the loations of the stimulating and recording electrodes is given in Fig. 4(*a*). The earthed electrode was simultaneously the recording electrode and the cathode. Square pulses of different durations from a pulse generator whose internal resistance was over 3 MΩ were used for stimulation.

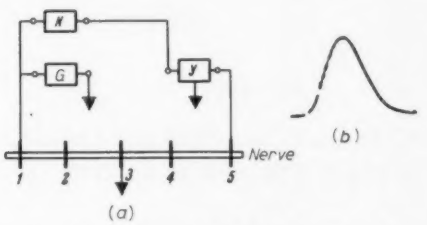

FIG. 4. *a*—Electron potential compensation layout. This is explained in the text. *G*—current generator; *y*—amplifier with differential input; *K*—compensating voltage generator; 1, 2, 3, 4, 5—electrodes.
b—Oscillograph recording of the local response obtained as a result of electron potential compensation. The start and end of stimulation are denoted by capacitance spikes. The duration of stimulation was 300μ sec.

Compensation of the electrotonic potential and recording of the local response were, in practice, accomplished in the following way: sub-threshold intensity stimulation was applied to the nerve. During this, the electrotonic potential alone was visible on the screen of the oscillograph. The compensating voltage generator was then switched on, and the shape and amplitude of the pulse fed into the other amplifier point of entry were made such that a smooth line was seen on the screen. The spikes remaining, though reduced to a minimum, were caused by the stray capacitances of the circuit; they did not impede observation even when short-duration stimuli were used (Fig. 4(*b*)). Compensation was not disturbed when the intensity of stimulation

was altered, since the voltage fed into the compensating voltage generator was proportional to the stimulating current. This made it possible to study the local reaction of a nerve under stimulation of any intensity.

SUMMARY

(1) The various circuits for recording the localized response of a nerve used previously by other authors [1, 4, 6, 8] are examined in this article, as well as those used for the first time in my work.

(2) Bridge diagrams are analysed and the deduction made that they are unsuitable for quantitative study of sub-threshold localized responses.

(3) The basic requirements made of procedures for recording localized responses are set forth.

(4) A new method of compensating the electrotonic potential, based on feeding a compensating voltage into one of the input points of a differential amplifier, is described.

Translated by J. H. DIXON

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THE ROLE OF BIOELECTRIC POTENTIALS IN THE INTAKE AND TRANSFER OF MATERIALS IN PLANTS*

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A NUMBER of important details concerning metabolic bioelectric potentials (b.e.p.) has been revealed by means of plant electrophysiology. A large number of investigations has shown the close link between b.e.p.s and the basic vital functions of the organism: respiration, photosynthesis, etc. [1–4]. The great variability of b.e.p.s under the influence of different external factors has also been demonstrated [4–7]. At the same time, the role played by these potentials in the vital processes of plants has still not been explained experimentally.

In 1955 [8] we started an investigation of bioelectric potential differences (p.d.s) in tomatoes in relation to the effects of hetero-auxin (HA) and α-naphthilacetic acid (ANA), as shown by the following diagram:

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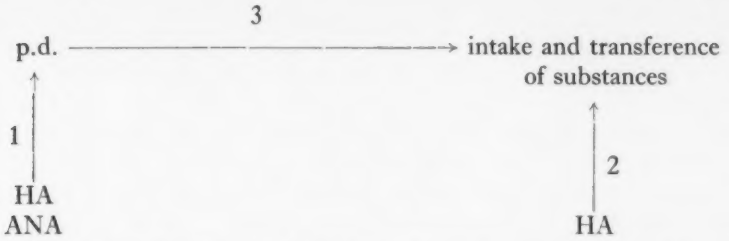

It was established that the p.d. and ^{32}P intake of tomatoes are increased by stimulating doses of these substances and decreased by inhibiting doses. Hence, links 1 and 2 in the above diagram were revealed. The results obtained led us to suggest the possible existence of a close relationship between the intake and transfer of substances in plants and their p.d.s (link 3).

The probability of a link between p.d.s and the processes of intake and transfer in plants has been noted in the literature on more than one occasion. Lundegardh, for example, suggests that potential gradients are very important both in metabolism and the transference of substances within the organism and for ionic adsorption and excretion [9]. According to Lund, the surface potentials of plants may carry out electrical work in a number of cellular processes, including the selective transport of ions [10]. The concept of plant nutrition as an electrical phenomenon is developed by Breazeale *et al* [11].

In view of this, we decided to concentrate on the role of p.d.s in the intake and transfer of substances in plants. The work was carried out in three experimental stages.

First stage. This stage involved experiments aimed at confirming our earlier findings [8] on the connexion between the effect of HA on the intake of ^{32}P by tomatoes and their p.d.s. Besides repeating our experiments with ^{32}P, which gave confirmatory results, we carried out a simultaneous study of the p.d.s of "Bison" tomatoes (water cultures) and their intake of ^{14}C (as $NaH^{14}CO_3$) when the roots were treated with HA. Data concerning one of two experiments giving analogous results are given in Table 1 and in Fig. 1. These results show that treating the roots of the tomatoes with a stimulating dose of hetero-auxin (5 mg/l.) increases their p.d.s, at the same time leading to a marked rise in ^{14}C intake by the roots, increasing its content in all parts of the plant in comparison with the control. An inhibiting dose of HA

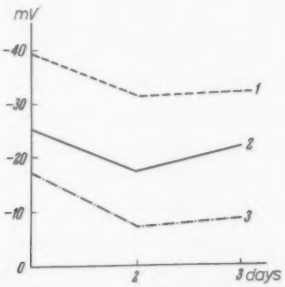

FIG. 1. Potential differences between the tip and the base of tomato stems after treating the roots with hetero-auxin. 1—HA 5 mg/l.; 2—water control; 3—HA 400 mg/l.

(400 mg/l.) has the opposite effect. The changes in electrical polarity noted after treating the roots with HA are also accompanied by polar changes in the distribution of the ^{14}C within the plant. Thus, if the ratios of the activity of the ^{14}C (counts/min) at the top of the plant (upper parts of leaves + leaf bases + upper stem) to that in the lower parts (stem base + roots) are calculated, the following figures are obtained: control, 0·160; HA (5 mg/l.), 0·363; HA (400 mg/l.), 0·053. This ratio reflects to some extent the degree of upward movement of ^{14}C in the plant. For this reason, we were able to conclude from these figures that a stimulating dose of HA causes an increased potential difference and facilitates transfer of ^{14}C from the lower parts of the plant to the higher parts, whereas an inhibiting dose produces the opposite effect.

Hence, the experiments with ^{14}C fully confirmed the relationship established earlier between changes in p.d.s and the intake and transference of ^{32}P (as $KH_2{}^{32}PO_4$) in tomatoes under the influence of stimulating and inhibiting doses of HA. It may be suggested that this relationship is based on one of two circumstances: (1) the generation of electrical energy and the intake and transfer of substances are not interdependent but are equally affected by HA; (2) there is a direct connexion between the two processes, whereby a change in one, caused by the action of HA, brings about a change in the other.

It should be emphasized, however, that these two possibilities are not exhaustive. Part of our further investigation was to establish a direct link between electromotive force (e.m.f.) and the intake and transfer of substances in plants. On analysing the published material dealing with this subject, we considered it expedient to suggest as a working hypothesis that the work carried out by the plant in accumulating and transferring substances has the e.m.f.s of cells and tissues as one of its sources of energy.

The methodological approach which we adopted in the subsequent experiments consisted of a study of the intake and distribution of ^{32}P by tomato and wheat shoots

TABLE 1. THE EFFECTS OF TREATING THE ROOTS WITH HETERO-AUXIN ON THE INTAKE AND DISTRIBUTION OF ^{14}C IN "BISON" TOMATO PLANTS (COUNTS/MIN PER MG DRY MATTER)

Experiment	Leaves, tip	Leaves, base	Stem, tip	Stem, base	Roots	Total activity
Water control	25	27	27	69	423	571
HA, 5 mg/l.	83	107	172	290	708	1360
HA, 400 mg/l.	0	0	5	10	283	298

with simultaneous alteration of their electrical polarity. The difficulty in setting up these experiments lay in selecting an external factor which would effectively alter the value of the e.m.f.s of the plants while having a minimum effect on all the other aspects of their activity. In solving this problem we were guided by Lund's statements on the two ways of changing the inherent e.m.f.s of plants: the indirect and the direct [10].

Second stage. This stage of the investigation consisted of a study of the intake and distribution of ^{32}P in tomato shoots whose electrical polarity had been indirectly affected. The indirect alteration of the e.m.f.s had been achieved by local treatment

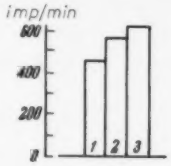

FIG. 2. ^{32}P intake by tomato shoots after treating with a 5×10^{-6} solution of $CdCl_2$. 1—after treatment of tips; 2—control; 3—after treating bases.

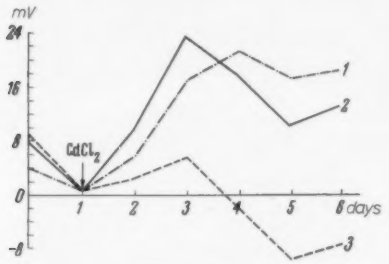

FIG. 3. Potential differences between tip and base of tomato shoots after treating with a 5×10^{-6} solution of $CdCl_2$. 1—after treatment of tips; 2—control; 3—after treatment of bases.

of the shoots with a 5×10^{-6} solution of $CdCl_2$, which was applied several times a day to an approximately 5 mm section of the tip or the base of the shoots. The use of $CdCl_2$ for affecting the generation of electrical energy by living tissues is well recognized in the literature [12]. We used untreated plants as controls. The tomato shoots were grown in Petri dishes on silica sand with a complete Knop's nutritive mixture (pH 6·3) until two leaflets appeared. The plants were treated with $CdCl_2$ over six days, and measurements of their p.d.s were made during this time (between the tip and the base of the shoots) by the technique which we used previously [13]. After this, 10 ml of Knop's mixture, diluted four times, containing ^{32}P with a total activity of 3 μc, were put into each Petri dish, and after 24 hr the plants were removed and fixed. The impulses from the ground, dried material were counted on a B-2 apparatus with an AS-1 tube. Data regarding the changes in p.d.s and ^{32}P activity in one of the experiments are given in Figs. 2 and 3. Comparison of these figures shows that treating the bases of the tomato shoots with a 5×10^{-6} solution of $CdCl_2$ caused an alteration of their electrical polarity to the negative side and an increase in their overall ^{32}P content compared with the controls, while the opposite applied for the plants whose tips were treated with $CdCl_2$. If the various parts of the experiment are placed in order of increasing negativity (or decreasing positivity) of the p.d.s ("$CdCl_2$ to tips"–"control"–"$CdCl_2$ to base"), the same order applies to the increase in overall ^{32}P content. That is, a greater shift of the p.d.s of the shoots to the negative side corresponds to a greater intake of ^{32}P by the shoots. It should be noted that increased ^{32}P intake by the plant as the negativity of its p.d. increases was also

established in our earlier experiments [8]. This fact is of great interest, and demands careful experimental interpretation. It may be suggested that it is to some extent linked with the electrical properties of the form in which phosphorus is transported within the plant.

With regard to the reason for the electrical effects of local treatment of the shoots with a $CdCl_2$ solution, the following assumptions may be put forward. The 5×10^{-6} $CdCl_2$ solution causes an increased electro-positivity of the area of tissue to which it is applied. Since, in our experiments, the tips of the plants were positive with respect

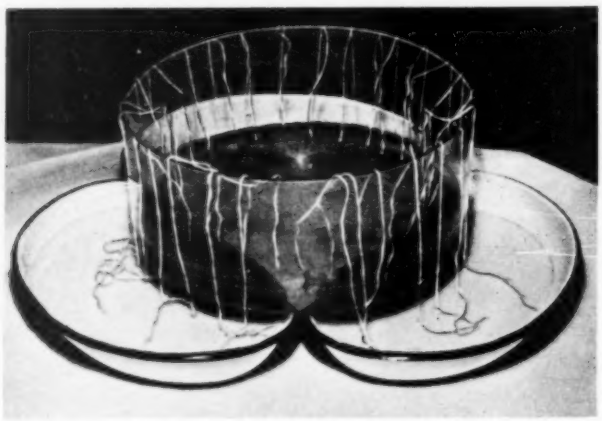

FIG. 4. Dish with shunted wheat shoots.

to the bases, treating the latter with $CdCl_2$ solution naturally led to a shift of the p.d. in a negative direction. The application of the $CdCl_2$ solution to the positive tip of the plant must have caused an increase in its positive potential probably with no change in the potential of the base of the shoot, thus changing the value of the p.d. in a positive direction. All this is mainly hypothetical and therefore needs experimental confirmation.

Analogous experiments were set up frequently, in particular with the additional treatment of tomato shoots with stimulating and inhibiting doses of hetero-auxin, and in most cases gave similar results.

The method of acting locally upon a subject by means of a solution of $CdCl_2$ (or of some other chemical substance) in order to change its overall electrical polarity will undoubtedly be very useful in future investigations. This method, however, besides altering the generation of electrical energy, may cause disturbances in a number of the other vital processes in the organism, which must be taken into account. Our next task was to carry out experiments involving a technique which was more or less free from this disadvantage.

Third stage. In this stage we studied the immediate connexion between the electrical polarity of plants and the intake and transfer of substances, using a direct method of affecting their biopotentials. Lund notes two possible ways of directly affecting plant e.m.f.s: (1) applying an external potential difference; (2) decreasing

the resistance of the medium surrounding the plant tissues (shunting). We have concentrated on the second means of changing p.d.s, since the first is in fact indirect and is rather primitive.

It has been shown by a number of authors that the application of a fluid conductor (tap-water etc.) to a section of plant tissue causes a flow of bioelectric current [10, 14, 15]. In a short-term experiment, other vital processes are probably very little affected by this.

The experiments were set up as follows (Fig. 4). Tomato and wheat shoots were grown separately around the edges of two shallow dishes in silica sand with Knop's nutrient mixture (pH 6·5). When the shoots had reached a height of 2·5–3 cm, fine wicks were laid along their axes, soaked in (1) twice-distilled water (a poor conductor), and (2) tap-water (a good conductor). In the work with the wheat shoots we also used a 0·01 N solution of NaCl (a good conductor). Care was taken that the lower end of the wick was about 0·5 cm above the level of the sand. The experiments were

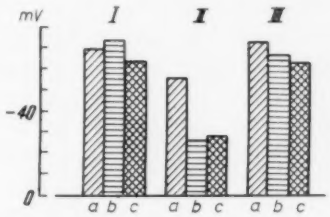

FIG. 5. The shunting effect of fluid conductors on the p.d.s of wheat shoots. I—original p.d.; II—p.d. after applying conductors; III—p.d. after removal of conductors. *a*—twice-distilled water (control); *b*—tap-water; *c*—0·01 N solution of NaCl.

arranged in such a way that the wicks were always damp. The experiments with both tomatoes and wheat involved 10–12 plants for each part of the experiment. After 12 hr, 15 ml of four times diluted Knop's mixture containing ^{32}P with a total activity of 10 μc were added to each dish; 6–7 hr after this, the wicks were carefully removed, the plants were divided into three parts (tip of shoot, base of shoot and roots) and fixed. The material was dried in a silica crucible, and a weighed sample placed on an aluminium base. The samples were counted on a B-2 apparatus with an AS-1 tube. Data on one of three experiments giving similar results are given in Table 2.

TABLE 2. INTAKE AND DISTRIBUTION OF ^{32}P IN TOMATO AND WHEAT SHOOTS IN CONNEXION WITH SHUNTING OF THEIR POTENTIAL DIFFERENCES (COUNTS/MIN PER MG DRY MATTER)

Fluid conductor	Tomatoes			Wheat		
	Leaves	Stems	Roots	Tip of shoot	Base of shoot	Roots
Twice-distilled water (control)	97	213	1935	93	779	1699
Tap-water	48	116	1282	42	364	1008
0·01 N NaCl solution	—	—	—	49	497	1404

These results show clearly that the application of fluid conductors such as tap-water or a 0·01 N solution of NaCl to the plant causes a considerable decrease in the ^{32}P intake of both the aerial parts and the root of the shoots in comparison with the experiments where the wicks were soaked in twice-distilled water. It must be emphasized that the plants in the different experiments did not differ at all in their external appearance.

In order to make sure that the p.d.s of the shoots were in fact shunted in our experiments by the 0·01 N NaCl solution and the tap-water, we made special measurements of the p.d.s of wheat shoots. The experiment was set up as above. The p.d.s were measured before shunting, after shunting and after removal of the wicks. The shunting lasted for 1 hr. When measuring the p.d.s of the shunted plants the plates of the electrodes did not touch the damp wicks. The results of one of four similar experiments are shown in Fig. 5. This shows that the original value of the shoot p.d.s (about equal in all three parts of the experiment) was changed very little by the effects of the twice-distilled water, whereas tap-water and 0·001 N NaCl solution decreased it considerably. In all cases the shoot p.d.s returned to the original level soon after the wicks had been removed, indicating that these changes in p.d. had obviously not been simply the result of metabolic disturbances but were caused mainly by leakage.

It is convenient to shunt for 1 hr when measuring the p.d.s, but this period does not correspond with that employed in the experiments for studying ^{32}P intake and distribution (18–19 hr). To obtain completely comparable conditions we set up a special experiment in which wheat shoots were shunted for 18–19 hr and their p.d.s measured. The results of this experiment were completely analogous with those obtained in the previous experiments.

Hence, our experiments showed that placing wicks soaked in 0·01 N NaCl solution or tap-water against the shoots caused a diminution of their surface metabolic potentials and at the same time caused a marked lowering of their ^{32}P intake. This leads us to assume that the e.m.f.s of cells and tissues may play a definite part in the intake and transference of substances in plants. We suggest that these results may provide a new approach to the explanation of the mechanism of these processes. T. G. Kitaeva made a special laboratory check on our experimental data, and drew similar conclusions.

CONCLUSION

Although the experimental material given above is in many cases preliminary in character and needs confirmation, the conclusion drawn from it regarding the possible significance of plant e.m.f.s in the intake and transfer of substances is a very probable one. At the moment, however, the mechanism of this phenomenon is not clear. This is mainly because very little work has been done either on the mechanism of the intake and transfer of substances or on the factors involved in the development of plant e.m.f.s. The generation of electrical energy and the accumulation and transfer of substances is undoubtedly linked primarily with the metabolic processes of the whole organism. A study of the literature shows that this link is probably achieved not by one but by several means. In our opinion, this enables us to assume that the electrical polarities of cells and tissues may, in various ways, take part in the absorption and transport of substances.

The following types of activity are quite possible:

(1) *Ionic adsorption.* This is by nature an electrical process. A number of interesting papers by Sabinin, Kolosov, Kursanov *et al.* [16–18] shows that both the intake and transfer of substances in plants may be achieved by means of ionic adsorption.

(2) *Electro-osmosis.* The idea that this process may take part in the transport of water and of the substances dissolved in it has been put forward in the literature [16, 19]. Stamm [20] established that certain conditions are essential for electro-osmosis to arise in the conducting vessels of plants. Bennet-Clark and Bexan made a neat attempt to establish experimentally the existence of electro-osmosis in plant cells and tissues [21].

(3) *Electrical transfer of ions.* This possibility is put forward mainly by Lund's school [10], although we know of no direct experimental investigation.

(4) *Electrophoresis.* It is very likely that this process may occur in a complex heterogeneous system such as living protoplasm. It is also possible that simply-organized substances, acting together with colloid particles, may be transported along with them by means of electrophoresis. There have been no experimental observations on this subject, however.

There may be other ways in which electrical energy is involved in the work of accumulating and transporting substances in plants. The study of all the possible ways in which it may be involved, with a concise account of the energy changes in the organism, is one of the biophysicist's most important tasks.

Finally, it should be emphasized that the conclusions drawn in this paper are based only upon changes in surface metabolic bioelectric potentials, since at the present time there is no means by which the e.m.f.s of intracellular or tissue systems may be determined. However, these changes have a definite value in that the surface e.m.f.s may possibly play an independent part in the vital processes, and their values reflect to a certain extent the internal electrical polarity.

The author would like to express his grateful acknowledgement to V. A. Guseva for her supervision of this work.

SUMMARY

(1) There is a definite relationship between the effect of hetero-auxin on e.m.f.s and the intake and transference of ^{32}P and ^{14}C in tomato plants. This leads to the concept of a possible close link between the electrical polarities of cells and tissues and the processes of absorption and transport of substances by plants.

(2) Alteration of the potential differences of tomato shoots by treating a small area of their tips or bases with a $CdCl_2$ solution leads to considerable alteration in the overall ^{32}P intake by the plant. A shift of the p.d. to the negative side is associated with a higher ^{32}P intake.

(3) The application of fine wicks soaked in 0·01 N NaCl solution or tap-water to tomato or wheat shoots causes leakage of their surface metabolic bioelectric potentials and considerably decreases their ^{32}P intake. This leads us to assume that e.m.f.s must play a definite role in the intake and transfer of substances in plants.

Translated by J. J. OLIVER

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EXPERIMENTAL STUDIES ON VARIOUS VISION SYSTEMS*

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1. THE subject of this paper is vision, but much of the discussion is readily extended to other sense organs or even to artificial information-handling devices. We will not depart from questions of vision, however; we only wish to point out that what we have to say here should not be considered as specific to the visual analyser.

Vision results from the visual system's reacting to light, and hence "to see" must always mean "react to light". If the eye reacts to light; it sees, otherwise not. This statement is so obvious that it is not usually explicitly formulated, but the way some problems are handled by some workers leads one to conclude that the interpretation of the concept "to see" is the object of the investigation. The above formulation also requires qualification; simple reaction to light is insufficient, "to react in different ways to differing radiations or combinations thereof" would be better.

Vision provides objectively correct information in so far as it enables one to differentiate objectively different situations. When particular vision systems (e.g. in animals) are being studied, the primary problem is to determine what objectively

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correct information the system can provide. Thus in such work "to see" should be taken to mean the capacity to differentiate objects, one from the other.

"To see" has several distinct meanings, depending on whether radiations or non-self-luminous objects are intended. "To see light of a given wavelength" usually means to differentiate that light from darkness, or to differentiate the light in question from the complete absence of light of any kind. Objects which are not self-luminous are not visible in the dark (are not distinguishable from darkness). To see such an object (differentiate its presence from its absence) it must be differentiated from its surroundings or background. The protective colours of animals (which make their presence inappreciable) are always similar to their backgrounds. A given object will be visible on one background, and not on another, to a given vision system. No answer can be given as to whether an object is seen or not unless the background is also stated.

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With any particular object, the faculty of recognizing it, i.e. differentiating it from anything else seen previously, is equally important. It is commonly accepted that the conditions where an object is only seen to be present are distinct from those where it is recognizable. From our viewpoint this difference must be stated as that in the first case the mere presence or absence are to be differentiated; whereas the capacity to differentiate the particular object from others (which must also be stated) is involved in the second. This formulation of vision as the capacity to differentiate some objective situations from others has the following importance in experimental studies. In considering particular problems of vision, one should remember that the possibility of an object (including radiations or combinations thereof) being seen implies that not only must the object be stated, but that the object or objects from which it has to be differentiated must also be stated. If the latter are not given, the problem is undefined. Some of the assumptions discussed later on will serve as illustrations and will demonstate that the problem must be posed as above. It will be shown in these instances that inaccurate formulations result in incorrect deductions and in misunderstandings.

The famous German entomologist Frisch and his school have shown that bees' eyes are insensitive to long-wave (red) radiations, but sensitive to the near ultra-violet, to which the human eye is insensitive [1]. Frisch and others considered the failure of bees to see red light difficult to reconcile with the fact that bees diligently seek out red poppy flowers and find them easily. Frisch supposed that the poppy flowers reflected ultra-violet light (visible to the bees); this was accepted without question by others as explaining how bees find poppy flowers.

Although the facts are correct, the interpretation is undoubtedly erroneous. The work of Frisch [1] and Kühn [2] showed that bees cannot differentiate long-wave radiations from the complete absence of light. To find the poppy flowers they do not have to differentiate them from darkness but from their surroundings, i.e. primarily from green grass. Frisch's experiments showed that bees differentiate the green light reflected by grass from darkness easily; hence to a bee any red flower must stand out sharply on a background of green grass, no matter whether the flowers reflect ultra-violet light or not.

Many would be inclined to say that red flowers appear black to the bee when seen against green grass. This supposition is particularly suggestive because everyone is

aware of such effects from his own visual perceptions. But we should avoid such expressions because we know nothing of the sensations animals experience, and moreover cannot correctly describe them in terms of human vision. All we can know about vision in animals is what is found from their objectively detectable reactions to objectively determined external stimuli. In this way we can investigate what objects an animal can differentiate. This is sufficient for our purpose here. Comparisons with human sensations are impermissible in describing such phenomena; they only result in misunderstandings.

This error on the part of entomologists not only resulted in an unnecessary search for an explanation of how bees see poppy flowers, but also left unexamined the problem of how they find orange and yellow flowers. The spectral reflection curves show that yellow and orange objects differ from greens of various shades mainly in reflecting in the red region. The former reflect red well, while the green absorb red strongly. Since the presence and absence of long-wave radiations are the same to bees, they should find it difficult to locate orange and yellow flowers in green grass. Frisch found, using variegated objects, that bees could possibly differentiate yellow from green. A number of arguments could be put forward here, but we do not wish to be involved in speculation as to how bees find yellow and orange flowers, since the studies were incomplete.

The topic is far from exhausted by this example, because the same sort of error has been made again and again. For instance, some cast doubt on whether the power to perceive ultra-violet light is of value to insects, since most flowers reflect but little in this spectral region. The data available show that the ultra-violet reflection coefficients of various flowers differ quite markedly from one another and from the value for green grass.*

We now consider, again for insects, the minimum dimensions at which objects are differentiable. This is usually referred to the resolving power of the system in the same way as for optical instruments or photographic films. Insects' eyes are built up from facets, each facet reacting to the light incident on it as a whole, all image details of angular dimensions less than those of one facet being lost. As regards the resolving power, one such facet is equivalent to a single grain in a photographic emulsion or the image patch produced by an optical system from a luminous point. The resolving power of a compound eye is determined by the angular density of its facets (number of facets per unit solid angle). This density is always readily found; values have been published for various insects.

Some studies on insect behaviour have, however, appeared to give results contradicting such calculations; thus dragon-flies feed on small insects, which they can see from several metres away.† The angular dimensions of such an insect at this distance are considerably less than those of one facet. But does this contradict the argument as to the resolving power of a facetted eye ? The problem is really one as to what the eye has to differentiate in resolving power experiments and as to what in detecting small insects.

* See Mazokhin-Porshnyakov [3] for some interesting ultra-violet photographs. The same author [4] has published a paper on the red poppy question which gives arguments similar to those presented here.

† They also feed on fine objects on the ground, showing that they see them. They might hear the insects flying, if in the air.

Suppose we have two black points very close together; a system of high resolving power can record these as two distinct points separated by a brighter gap. The two points coalesce in worse systems. Here two objects have to be distinguished from one. To do this the two objects plus the gap between them must occupy at least three independent facets. Any combination of stimuli to two adjacent receptors can be produced by projecting on them one dark object of suitable size and position. Thus two receptors are insufficient to distinguish one object from several. If three receptors are involved our two dark objects will result in the centre receptor's always being more brightly illuminated than the edge ones, which can never occur with one object. Hence the three receptors differentiate two objects from one. But when a dragon-fly "sees" a midge, it has only to detect that some object (midge) is present in the field of view, i.e. differentiate one object from none. This is quite a different problem.

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If a dark object (or one merely differing from its background) appears within the angle of view of one facet the amount of light received falls, so the presence or absence of the object can be differentiated. If the contrast sensitivity in the receptor is sufficiently high the object will be detectable even if its angular dimensions are much less than those of one facet. This faculty depends on how the intensity alters when the object appears and on the contrast sensitivity of the receptor, but not at all on the angular density of facets or the resolving power of the eye. The dimensions of the object are only indirectly significant, in so far as the intensity change between object present and absent decreases with the size of the object.

This example demonstrates how it is that we can see a star in the sky. The fixed stars are clearly seen, in spite of their very minute angular dimensions, but multiple stars are not differentiated from single even though their separations are much greater than their diameters. In the first case only the presence or absence of a star has to be detected, in the second two have to be differentiated from one.

In signalling problems much work has been devoted to determining the angular dimensions required for various geometrical shapes to be recognized correctly. Very often the tests are conducted by presenting a shape (a square, say) to the subject and determining the maximum distance at which it is correctly recognized. Road signs are often selected from such tests. The results sometimes show large divergences and in others quite good reproducibility. The cause, we believe, should be sought in the problems not always being correctly posed; it should not be posed as "from what distance can a circle be recognized ?" but as "at what distance can it be distinguished from (say) a square (or other precisely defined and previously stated object) ? ". If the choice involves similar shapes it is made more difficult, and conversely. Although this has sometimes been remarked on in the literature, the correct conclusions have not always been drawn.

If the subject knows what shapes have to be differentiated the results are more clear-cut than if not, but in the latter case most subjects give generally concordant results, a square and a circle being correctly named at greater distances than an oval for instance. It might at first sight seem that such experiments show that we can ask whether a shape will be recognized without stating the choice of shapes to be differentiated, but such an approach is incorrect. In all such cases some choice of shapes from which to make his selection is always at the subject's disposal; if they are not prescribed in advance the basis of the test is undermined.

When a subject is required to recognize certain geometrical shapes in the laboratory the surroundings themselves indicate that the shape will be a geometrical one, and, when called upon to name a shape, the subject will tend to name the simplest. For instance, if conditions are such that the circle and oval cannot be distinguished, the name given is very rarely "oval", but very frequently "circle". This does not mean that the oval is "less recognizable" than a circle, but only that to most people a circle is "simpler". Another example is that of someone being called upon to name some remote object (of nature unknown) in an ordinary setting; the answers will usually be such as "man", "house", "horse", etc., and not "circle", "triangle", "square". The different observers will tend to agree, but this does not imply that geometrical shapes are less well distinguished in a natural setting than in the laboratory. The surroundings inevitably supply a more or less definite selection of objects, of which the observer unconsciously selects the most appropriate. Agreement between observers is merely due to their experiences in daily life being comparable, which implies that the objects they expect to see in a given setting are similar.

We may wonder whether it is possible to perform a test in which the surroundings suggest nothing; it is very doubtful whether this is possible. It is still more doubtful whether it could be proved that such conditions were observed. But it is found that the less the conditions suggest a selection of objects which may be expected to appear, the worse the reproducibility for a given observer and the greater the discrepancies between observers.

As regards recognition of various shapes, or more precisely of various objects, much could be learned from observations on how people recognize objects in a normal or unusual setting. We will not deal with examples which anyone can draw on from his personal experience.

We have shown, using several examples, how our proposal is of real assistance in solving particular problems; our thesis being that to see any object implies always to differentiate it from other precisely defined objects. Only if all the objects from which the given one has to be differentiated are stated does the problem have the degree of definition required for its solution.

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AN ELECTROPHYSIOLOGICAL INVESTIGATION OF THE EFFECTS OF BRIEF STRONG FLASHES OF LIGHT ON THE RETINA AND BRAIN OF MAN AND RABBITS*

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THE need to study the effects of brief strong flashes of light on the retina and brain is dictated by both theoretical and practical considerations. The various biophysical methods of investigation are very useful in this respect, since they are objective and can give comparable results both in human subjects and in experimental animals.

In the literature at our disposal we can find only isolated papers devoted to the study of electroretinogram (e.r.g.) changes during brief strong illuminations. Cobb and Morton [1] and Rendahl [2], who studied retinal potentials in man caused by flashes from a powerful vapour discharge lamp, described a considerable increase in the initial negative deviation of the e.r.g. (peak "*a*") as being the main distinguishing sign of the retinal effects of ultra-strong visual stimulation.

In preparing this paper, we were interested both in investigating the retinal potentials during the actual flash and in studying the electrophysiological indicators, which may provide some idea of the dynamics of the restorative processes in the retina after the flash, and also of the changes in the functional state of the central nervous system induced by strong flashes of light.

TECHNIQUE OF INVESTIGATION

The investigation involved 15 healthy persons aged from 20 to 30, and 10 rabbits with electroencephalograph electrodes permanently *in situ*. The positions of the subject and the instruments are shown in Fig. 1. The source of strong brief flashes of light was an ISS-250 impulse lamp with a flash energy of 250 J. The flashes lasted for about 1 msec, and the amplitude of the light flux perpendicular to the axis of the lamp was 400,000 candles. The lamp was situated either 1 m or 2 m from the eye of the subject. Light from an instrument producing ordinary intermittent illumination was focused on to the same eye. The e.r.g. obtained under these conditions was taken as an indication of the functional state of the retina at various times after the flashes. Our apparatus for producing intermittent illumination consists of a hollow cylinder driven round by a friction drive from a synchronous motor. Inside the cylinder are fixed a 76 W electric light bulb and a lens which focuses the light on to the eye of the subject. The cylinder can be moved by a ratchet so that the light from the bulb falls upon the eye through different numbers of openings at various levels of the cylinder (4, 8, 16, 32 openings). Thus the frequency of the

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intermittent illumination can be changed without changing the duration of each flash. The frequency and duration of the flashes can be changed by moving the friction drive nearer to the axis of the cylinder. This instrument allows for a flash frequency of from 0·5 to 90 c/sec, with a duration of 0·01–0·06 sec.

Careful screening of the supplies to the lamps and earthing of the screens exclude the appearance of artefact biopotentials on the trace at the time of the flash and allow reliable recording of the e.r.g. and electroencephalogram (e.e.g.) at this moment.

The biopotentials were recorded on an apparatus consisting of six independent amplifier circuits and a six-channel oscillograph. All the amplification circuits were compensated, with long constant intercascade intervals (RS-4). Simultaneous e.e.g.s

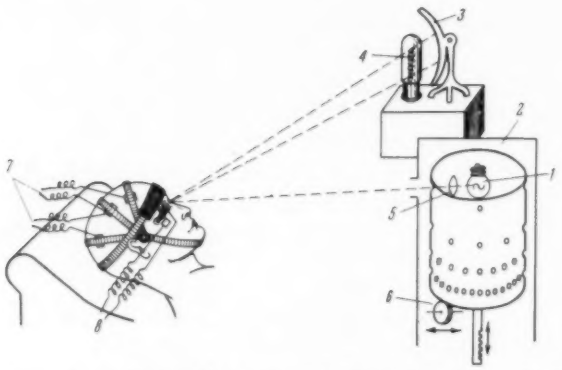

FIG. 1. Positions of the subject and apparatus in the experiments. 1—76-watt bulb; 2—instrument for producing intermittent illumination; 3—concave mirror; 4—1SS-250 impulse lamp; 5—lens; 6—friction drive; 7—e.e.g. electrodes; 8—e.r.g. electrodes.

and an e.r.g. were recorded by several bipolar leads. One amplification channel served as a marker of the light stimuli, with the aid of a photocell incorporated in the amplifier input. The e.r.g. leads consisted of a precorneal electrode in the form of threads soaked in normal saline and passed through a silver tube held in the correct position by a brow-band. The other e.r.g. electrode was placed on the skin over the cheek bone. The conjunctiva was anaesthetized with 0·5% dicaine. To avoid blinking, a light eyelid retractor was applied to the eye. The subject's eye was fixed on a luminous point on the impulse lamp, while the other eye was closed. The order of the experiment was as follows: after 2 min of darkness the eye was subjected to intermittent illumination with a duration of 0·05 sec and a frequency of 1 flash/sec. After some time the *b*-waves caused by this light reached a definite height on the e.r.g. A flash from the ISS-250 lamp was made against this background, and the retinal and brain potentials were recorded during the flash and for several minutes afterwards. Hence in each experiment we recorded the e.r.g. and e.e.g. of the ordinary intermittent illumination before the flash and also the changes in potential caused by the flash itself. Traces of some 300 flashes of the ISS-250 lamp were made in humans and rabbits.

RESULTS

Changes in retinal potential caused by brief, strong, flashes of light

In all the 250 e.r.g.s which we obtained from the 15 subjects, there are almost equal changes in retinal potential at the moment of the flash by the ISS-250 lamp, which enable us to note specific differences between the e.r.g.s arising during the flashes (Fig. 2 (B, D, F, H)) and e.r.g.s recorded during the ordinary intermittent illumination (Fig. 2 (A, C, E, G)).

On the basis of our observations, we were able to conclude, as were Cobb and Morton, that the chief difference in the retinal potentials arising during flashes of bright light (in comparison with the ordinary e.r.g.) is the appearance of an initial deep negative potential *a*. The traces made when the photokymograph is running

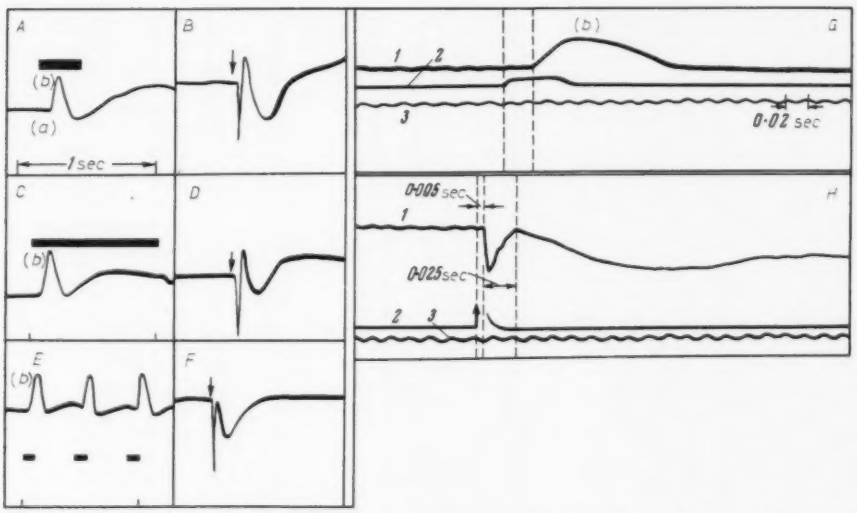

FIG. 2. Retinal potentials arising after ordinary illuminations (A, C, E, G) and after flashes by an 1SS-250 impulse lamp (B, D, F, H). A, B, C, D, E, F: traces made with slowly-moving kymograph; G and H—rapidly-moving. The ordinary illuminations are marked by a thick line, the bright flashes by arrows. G and H: 1—e.r.g. trace; 2—signals of illumination by photocell; 3—time signals, 0·02 sec.

quickly (Fig. 2 (H)) show the presence of a very short latent period before the negative deviation in comparison with the ordinary e.r.g., lasting only 0·005 sec; the duration of the initial negative deviation is 0·02–0·03 sec, and its amplitude reached 100–250 μV, i.e. it was many times larger than the *a*-potential in the ordinary e.r.g. Cobb and Morton, using an amplifier with a low constant interval which distorted the form of the slower potentials, could not give a proper description of the subsequent changes in potential.

The frequency characteristics of our amplifiers enabled us to overcome this difficulty. After the negative deviation of potential *a*, the e.r.g. shows a positive potential corresponding in duration and amplitude to peak *b* of an ordinary e.r.g.

By analogy with the ordinary e.r.g. we called this second positive wave *b*. As with the *b*-wave of the interrupted illumination, the size of the *b*-wave after a flash depends

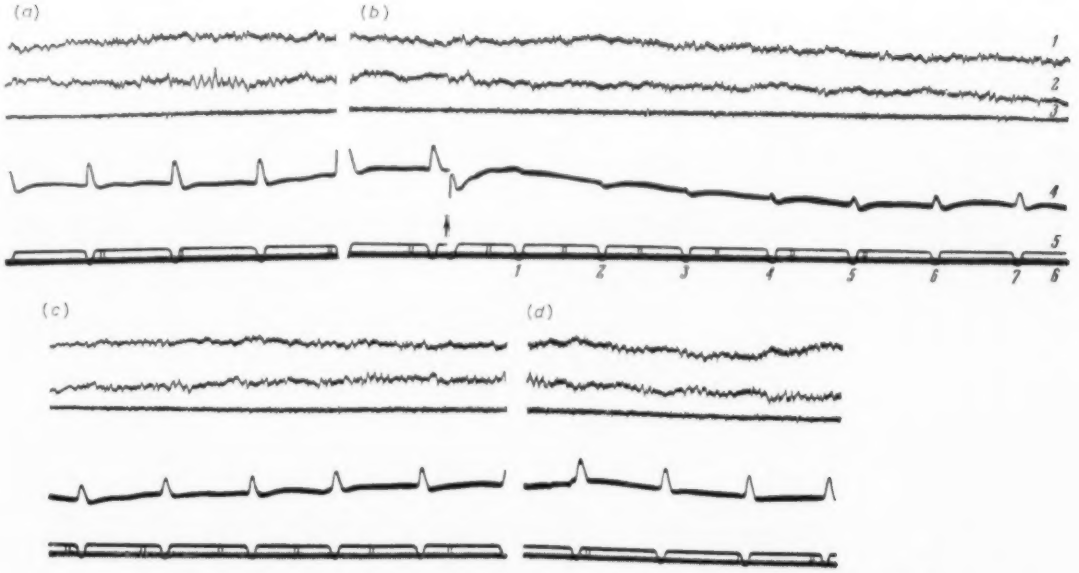

FIG. 3. From top to bottom, e.e.g. with temporo-occipital leads: 1—left side; 2—right side; 3—control trace of potential differences between neutral e.r.g. electrode and frontal region of the brain (this trace was made to exclude artefacts during flashes of the impulse lamp); 4—e.r.g. trace; 5—photocell signal. Moment of 1SS-250 impulse lamp flashes marked by arrows. A, B, C, D—successive traces.

upon the eye's previous state of adaptation. After dark-adaptation, the *b*-wave is increased, while after light-adaptation and especially after flashes made in daylight the *b*-wave is considerably smaller. The positive *b*-wave again becomes a negative wave c_1, which differs from the first negative deviation of the retinal potential in its duration (0·3–0·4 sec) and then after a more or less prolonged c_2 potential the e.r.g. curve returns to the original isopotential line.

The dynamics of the changes in the functional state of the retina after a flash (*e.r.g. findings*)

As has already been described in the section on technique, the subjects were in a dark room, with continuous (every second) brief illumination of the eye to obtain the e.r.g. Immediately after a flash the e.r.g. of the ordinary illumination disappeared for 1–2 sec, then responses gradually appeared; at first only the negative component of the e.r.g. appeared, the positive *b*-component arising some time afterwards (Fig. 3 (B), Fig. 4).

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If we plot the time (in seconds) after the flash with a logarithmic scale on the abscissa and the height of the *b*-wave (as a percentage of the original, before the flash) on the ordinate, we get a curve of the restoration of the height of the *b*-wave after a flash against time.

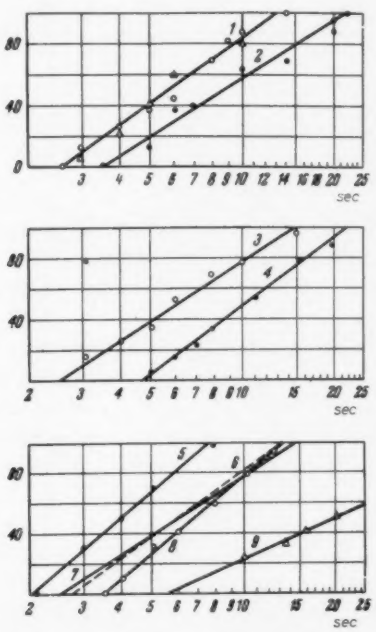

FIG. 4. Graphs of changes in e.r.g. *b*-peaks after bright flashes of light. The ordinate shows the amplitude of the *b*-peaks in percentages of the original value (before the flash). The abscissa is a logarithmic scale of the time after the flash in seconds. Above—changes in the *b*-waves in subject M with a flash–eye distance of: 1–2 m and 2–1 m. Centre—*b*-wave changes in subject S with flash–eye distances of: 3–2 m, 4–1 m. Below—curves of changes in the *b*-waves of five other subjects with a flash–eye distance of 2 m. The most rapid restoration occurred in subject A at 5 m and the slowest in subject F at 9 m.

Fig. 4 shows that in all the subjects the experimental points on the graph lay almost in a straight line. This means that the restoration of the height of the e.r.g. wave takes place proportionally to the logarithm of the time after the flash. Since the e.r.g. waves are absent for the first 2–4 sec (this must always be taken into account), the height of the *b*-wave after a flash as a percentage of its original height can be expressed by the equation

$$E = k \log (t/t_0).$$

This equation is approximately correct within the limits $t=t_0$ to $t=10t_0$, where k = proportional coefficient, t = time in seconds after the flash, t_0 = time after the flash during which the *b*-wave is absent from the e.r.g.

On the average, the value of t_0 with a flash-eye distance of 1 m is twice that with a distance of 2 m.

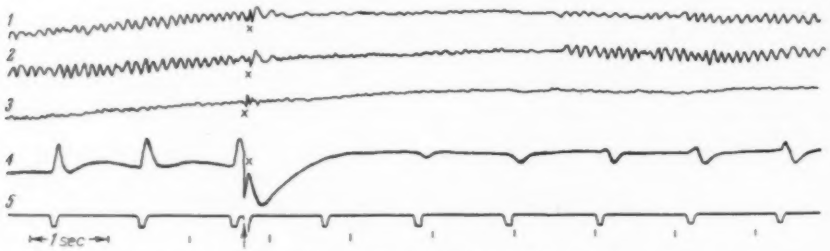

FIG. 5. E.e.g. and e.r.g. changes after flashes from 1SS-250 impulse lamp. Top to bottom, e.e.g. from temporo-occipital leads: 1—right side; 2—left side; 3—frontal lead; 4—e.r.g.; 5—photocell signals; below—time signals (1 sec). Subject M: distance from lamp to eye, 2 m; flashes marked with an arrow: primary reaction of retina and brain marked with a cross.

It may be assumed that the value of t_0 is directly proportional to the intensity of the flash and inversely proportional to the distance between the source of light and the eye, although a special study is needed to determine these relationships more accurately.

We examined our subjects repeatedly, over several years, and concluded that there are in fact some very slight variations in the *b*-wave restoration curve after a flash on different days of the experiment in one and the same subject, whereas individual variations among the different subjects may be very large and constant. In most of the subjects, the *b*-wave restoration curves after a flash were about equal and varied only slightly (Fig. 4, lower graph, Nos. 6, 7, 8). One of our subjects, however, an apparently healthy student F., showed a very slow restoration (Fig. 4, No. 9), while the retina of another subject, A., seemed to be less sensitive to flashes of bright light (Fig. 4, No. 5). These peculiarities remained unchanged over a whole year. The electrophysiological method obviously enables an objective estimate to be made of the sensitivity of the retina to bright light and of the individual variations in this sensitivity.

Electroencephalogram changes caused by flashes of bright light

The visual disturbances following a flash may result from changes at both the peripheral and central ends of the visual apparatus. We therefore associated the electroretinogram traces with a trace of the brain potentials (e.e.g.) in all the

FIG. 6. E.r.g.s and e.e.g.s of rabbits after flashes from impulse lamp 1SS-250. A: 1—photocell signal; 2—e.r.g.; 3—e.e.g. B: 1—e.r.g.; 2—e.e.g. C and D: 1—e.e.g.; 2—e.r.g.; 3—photocell signal. C—slowly-moving kymograph. D—the same at higher speed. In all the photographs the moment of the flash is marked by an arrow and the primary brain reaction by a cross. Below—time signals (1 sec). On trace A: 1—time signal (0·02 sec).

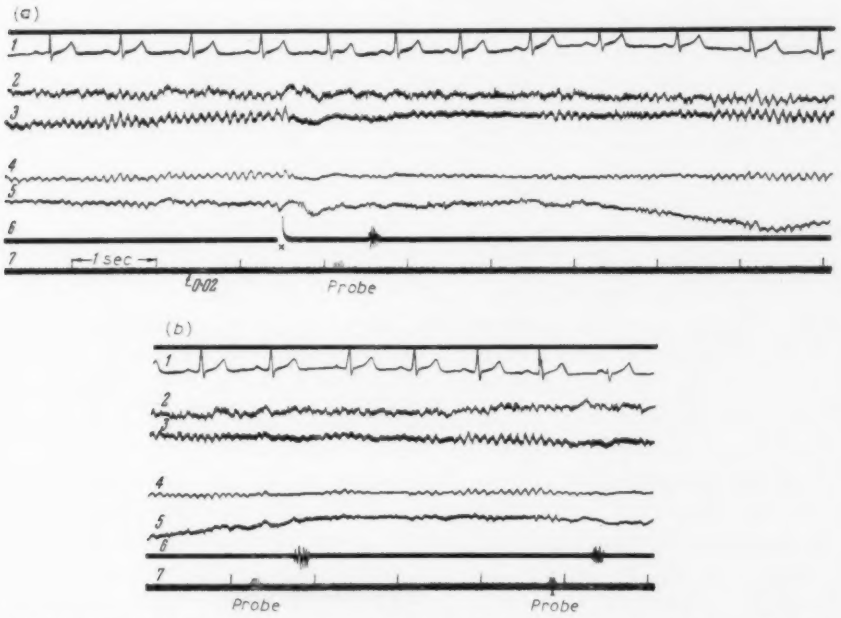

FIG. 7. Combination of flashes of light with study of conditional reflexes in man. Top to bottom, e.k.g., e.e.g. from bipolar leads. Fronto-temporal: 2—right; 5—left. Occipital: 3—right; 4—left. Track 6 shows simultaneously activation of the photocell and muscle oscillations (e.m.g.). 7—stimulus and time signal by a piezocrystal and small mirror. A—shows that the reflex after a flash of light (marked with a cross) has a shorter latent period than before the flash (B).

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experiments. It should be noted that the circumstances of the experiment did not favour the appearance of e.e.g. α-waves. The eyelid retractors, the expectation of the bright light, the open eye, the intermittent illuminations (1 per sec) did not produce the quiet, peaceful state without strong stimuli which is necessary for the appearance of a clear α-rhythm. Nevertheless, in 8 of the 15 subjects, on repeated examination, we were able to obtain well-marked α-waves from the temporal and occipital leads. In these 8 subjects depression of the α-waves lasting for 4–10 sec occurred after each flash, with a latent period of 0·1 sec (Figs. 5 and 7). Fig. 5 shows that the ordinary intermittent illumination did not cause any depression of the α-waves. More prolonged illumination causes a depression which differed from the above in having a longer latent period and a shorter after-effect.

In a number of the subjects, besides depression of the α-waves we noted another general brain reaction in the form of one or several frequent or slow deviations in potential (the "on" effect) arising 0·01–0·05 sec after the flash.

Hence, the changes in the e.e.g. after flashes of bright light were qualitatively similar to the usual responses to light stimuli, differing only in their shorter latent period and greater degree and duration.

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E.r.g. and e.e.g. changes (in rabbits) caused by flashes of bright light

Fig. 6 shows the e.e.g. and e.r.g. traces obtained in rabbits. The conditions under which they were obtained were identical to those employed for the human subjects. The bipolar electrodes for electroencephalography were first fixed permanently into the skull against the dura mater over the occipital region of the brain. The head of the animal was clamped into the required position during the experiment. The changes in the e.r.g. after the flashes were practically analogous in rabbits and in man. As in the human subject, a flash of light produces a complex e.r.g. consisting of an initial rapid negative potential *a* and subsequent slow potentials *b* and *c*, with the disappearance immediately after the flash of the e.r.g. response to ordinary intermittent illumination, followed by its gradual restoration proportional to the logarithm of the time.

In some rabbits the changes in the e.e.g. consisted of inhibition of the spontaneous rhythms (Fig. 6 (A)), while in others this inhibiting effect was absent. In all 10 rabbits, rapid two-phase responses (marked with a cross) similar to primary cortical responses after any other different stimulus were seen to arise in the occipital region a short time after the flash.

DISCUSSION OF RESULTS

The complex nature of the changes in retinal potential after flashes of bright light is apparently caused by the fact that strong illumination stimulates retinal elements having various stimulation thresholds, latent periods and durations of the excitatory process.

The initial negative deviation of the potential needs a high-intensity stimulus and is obtained only when the intensity of the light is high. This potential is maintained (though to a lesser degree) after light adaptation and even arises after flashes by the lamp in daylight. In its characteristics it corresponds to stimulation of the cone apparatus, having a high speed and a short latent period like that of the initial negative potential. The second, positive, deviation is markedly decreased after light-adaptation,

is absent in daylight and corresponds in its characteristics to the *b* wave of the e.r.g., which is associated primarily with the rod apparatus of the eye.

With weak flashes of light the positive *b*-wave predominates in the e.r.g.; the negative peak *a* increases with stronger flashes or after light-adaptation, while with very strong flashes (such as we used) negative components predominate in the e.r.g. The nature of the retinal action potentials is probably still more complex, as we have suggested in a paper written in collaboration with Lebedinskii [3].

In considering the dynamics of the changes in retinal potential after flashes of bright light, we would point out that with the gradual restoration of the original e.r.g. the first response to ordinary illumination is the appearance of a negative component, and only some time later does the positive *b*-wave appear. This means that both the rod and the cone components of the retina are affected by a bright flash of light, but the changes in the rod apparatus are more severe and prolonged than in the cones.

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On comparing the duration of the e.r.g. changes with that of the changes in e.e.g. rhythms, we may conclude that all the changes seen on the e.e.g. which are caused by the flash of bright light disappear before restoration of the e.r.g. to its original level. The biological significance of this fact is that after too bright a flash of light, within 0·005 sec of its appearance, changes have occurred in the retina which prevent further stimulation if the action of the light is continued, and in consequence protect the brain from the continued inflow of impulses.

The absence of any pathological changes in the activity of the cerebral cortex after the flashes of light in our experiments was confirmed by conditional reflex experiments (Fig. 7). A speech-motor technique was used to produce a good conditioned motor reflex to stimulation in our subjects. It transpired that in some of the subjects the latent period of the reflex was increased slightly after a flash, in others it was shortened and in a third group it was not altered. Postural reflexes and differentiation were maintained in all the subjects.

On this basis, we consider that in healthy persons the changes caused by short flashes of bright light derive more from the peripheral part of the analysing apparatus than from any effect on the cerebral cortex.

With flashes from a ISS-250 impulse lamp, which do not cause any pathological changes in the retina in man, ordinary methods of adaptometry and determination of visual activity could be used quite successfully. With stronger flashes of light, causing damage to the retina, experiments on human subjects and, consequently, all methods demanding subjective analysis, are excluded. It is only possible to study the effects of such flashes in animals. In this respect our results acquire a special interest, since they show that bioelectrical techniques can be employed and that the results obtained are comparable both in man and in warm-blooded animals.

SUMMARY

(1) Simultaneous electroretinogram and electroencephalogram traces were made in 15 human subjects and 10 rabbits after flashes from a ISS-250 impulse lamp.

(2) The changes in retinal potential caused by the flashes were found to possess characteristic distinctions from the normal e.r.g., consisting of a latent period of 0·005 sec, a rapid, deep initial negative potential *a*, a subsequent positive potential *b*, then a slow negative potential c_1, followed by an afterpotential c_2.

(3) The negative components *a* and c_1 were maintained after flashes in daylight and after light-adaptation and were apparently associated more with the cone apparatus of the retina, whereas the positive component *b* was sharply decreased after light-adaptation and increased in darkness, showing a greater dependence upon the rod apparatus of the retina.

(4) The normal e.r.g. did not appear if the eye was illuminated during the first few seconds after a flash, then the negative components of the e.r.g. reappeared, followed by the positive *b*-waves, increasing to their original (before-the-flash) value proportionally to the logarithm of the time after the flash.

(5) Brief flashes of bright light caused the same changes in the e.e.g. as does ordinary intermittent illumination (depression of the α-waves, "on" effect), differing only quantitatively. These are a shorter latent period and a greater degree and duration of the e.e.g. changes.

(6) The duration of the e.r.g. changes exceeded the duration of the changes seen in the e.e.g. A suggestion has been put forward regarding the defensive role of this retinal reaction to excessively bright light, protecting the brain from an excessive inflow of impulses.

(7) Bright flashes of light caused similar changes in the electrical potential of the retina in experiments involving human subjects and rabbits.

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A PHOTOGRAPHIC ILLUSTRATION OF THE RESOLVING POWER OF THE OPTICAL SYSTEM OF COMPOUND EYES OF INSECTS*

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WE know that the adult forms of insects possess two types of eye: complex or compound eyes (oculi) and simple eyes (ocelli). By function the compound eyes are true organs of vision, while the simple eyes apparently play the different part, as yet not sufficiently investigated, of additional photoreceptors [1, 2].

The compound eyes consist of a great number (20,000 or more) of separate optical units, the ommatidia, separated by pigment, each with its own dioptric and photoreceptor apparatus. With this type of structure, compound eyes function differently

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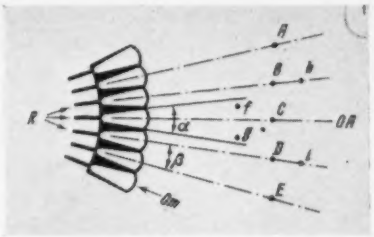

FIG. 1. Diagram of the apposition eye of an insect. *R*—rhabdome. *OA*—the optical axis of the ommatidia. *Om*—the dioptric apparatus of the ommatidia. α—the angle of vision of an ommatidium. β—the angle between the optical axes of ommatidia. The points marked *A*, *B*, *C*, *D*, *E*, *f* and *g* are points which the eye does not resolve. The points marked *h* and *i* are points which it does resolve.

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from those with a crystalline lens, such as the eyes of vertebrates. In 1826 I. Müller advanced the so-called mosaic theory for insects' vision. According to this theory each ommatidium provides an image of a small part of the object being viewed by the eye in the form of a dot, but all the ommatidia together form an image of the whole object made up of dots (the mosaic). The correctness of the mosaic theory for the vision of insects was subsequently confirmed experimentally by Exner [3] and other authors who studied the optical properties of compound eyes.

We know that the resolving power or visual acuity of vertebrates depends on the structure and sensitivity to contrast of the retina (the rods and cones) and the quality of the image created on it by the crystalline lens. The state of affairs is somewhat different with regard to the compound eye. Since only the rays which coincide with the optical axis of an ommatidium, or almost coincide with it, reach the rhabdome of that ommatidium, and the image which they form of a certain part of the object is perceived by insects as a luminescent spot, the quality of this image hardly affects the resolving power. Therefore the visual acuity of the compound eye in the perception of a stationary object is almost wholly determined by the "angular density" of the ommatidia, i.e. by the number of reacting ommatidia per unit of solid or plane angle, and by the sensitivity to contrast of the optic cells. I would emphasize that, as distinct from the assertions of the importance of the ommatidium angle of vision (the aperture angle α in Fig. 1) to resolving power which are widespread in works on the physiology of insect vision, the angle between the optical axes of the ommatidia is more important than their actual angles of vision (apertures).

The angle between the optical axes of the ommatidia determines the number of independent receptors recording an object of given angular dimensions, this being the angular density of the ommatidia. This statement is of material importance since the angles of vision of the ommatidia partially overlap, and are in consequence rather greater than the angles between their optical axes. The fact that the angles of vision of the ommatidia exceed the angles between their optical axes somewhat impairs the eye's visual acuity (owing to some degree of overlapping of images); however, it increases the sensitivity of the eye to light.

Consequently it can be stated, on the basis of the design of the compound eye, that the number of details which it can perceive separately in a stationary object depends on the angular density of the ommatidia and the angle subtended by the

object, which in turn depends on the distance from eye to object. Two points will, if the angular distance separating them is not less than twice the angle between ommatidia axes, always be perceived separately by this type of eye. Fig. 1 shows that points *h* and *i*, which are separated by an angle of 2β relative to the eye, will be perceived separately, while points *A*, *B*, *C*, *D* and *E* are beyond the limits of resolution, i.e. they cannot be distinguished from an unbroken line. Nor, in the same way, can points *f* and *g* be resolved.

The above relates firstly to the apposition eyes of day-adapted insects, and secondly to cases where the object being viewed is stationary relative to the eye. The resolving power of the superposition eyes of dark-adapted insects, in which each rhabdome receives rays not only through the lens of its own ommatidium but through those of a group of neighbouring ommatidia, is usually very low and is not solely determined by the angular density of the ommatidia. As regards the part played by the movement of an object relative to the eye, this can in some cases, where the sensitivity of the optic cells to contrast is high, increase the resolving power, i.e. the visual acuity may prove to be higher than that estimated by purely geometrical calculation. In perceiving moving objects, the resolving power depends not only on the optical properties of the eye but also on the physiological properties of the optic cells—their sensitivity to contrast—and on the critical flicker-blending frequency for the whole analysing apparatus [4].

The object of this article is to illustrate visually the capacity of insects to see, with relation to resolving the details of objects stationary relative to their eyes, or, more precisely, to reproduce the minimum amount of information which the dioptric apparatus of the compound eye is able to provide. I have taken the following as basic premises:

(1) For each ommatidium there is a separate nerve signal, independent of the signals from neighbouring ommatidia.

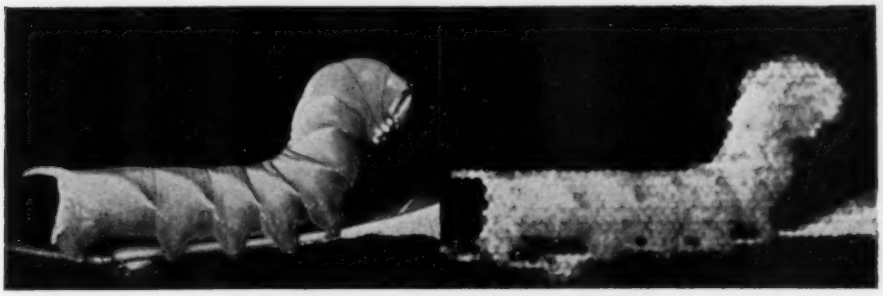
(2) The size of this signal depends on the total amount of light striking the ommatidium aperture.

(3) The light originating at any point in the eye's field of vision acts on one and only one ommatidium.

These premises are a well-known schematic thesis. In fact, as I have already said, the fields of vision of neighbouring ommatidia overlap to some extent; the stimulation of an ommatidium is not therefore entirely independent of that of its neighbours. This may to some extent reduce the resolving power, though not necessarily so since the amount of information is determined finally by the number of reacting ommatidia, i.e. by their angular density.

I aimed at obtaining on photographic paper images of various objects broken up into a number of separate elements equivalent to the number of ommatidia it took to produce a retinal image. Within each element the portion of the image had to be of uniform density when averaged over the parts of the image. This density represents the reaction of one rhabdome to the entire light striking the ommatidia.

The technical procedure was to project a negative image of the object through an enlarger on to a fine-grain opal glass plate, pressed closely against a perforated ebonite plate (a grid) which broke up the image into the separate elements. This plate, 3·5 mm thick, was drilled with cylindrical channels 1·5 mm in diameter and parallel



A B

FIG. 2. A—The caterpillar of the Privet Hawk Moth (*Sphinx Ligustri L.*). B—the image of the same caterpillar with the detail resolved by the eye of a bee from a distance of 9·5 cm.

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to one another, the distance between their axes being 2 mm. The grid was laid on the sheet of printing paper, covered by glass, on which the image was printed.

The result of printing using this appliance was as follows: the negative image of the object projected on to the opal glass by the enlarger was broken up by the individual channels into a fixed number of elements. The section of the opal glass covering each channel served as the source of light within that channel, illuminating a section of the photographic paper. Within each of the channels all parts of the projection image were neutralized to produce a small and uniformly illuminated spot on the printing paper. The resultant spot density represents the stimulation of one rhabdome. The glass placed between the printing paper and the grid permits blurring, on the print, of the shadow from the dividing walls between the grid channels. By altering the scale of the negative image projected on to the opal glass, it is possible to cover any number of channels by it, i.e. to imitate any correlation between the angular ommatidia density in an insect's eye and the angle subtended by an article being viewed by it.

Other attempts at the experimental reproduction of the image created by the compound eye optics of insects have been published. The relevant works are those of Exner [3] and Eltringham [5], who photographed images, formed by taking microscopic sections of the dioptric apparatus of insect's eyes and magnified by microscope, of objects. These photographs cannot, however, correspond to retinal images since the microscope also resolves details within each ommatidium which the insect's eye perceives simply as bright spots. They reproduce the images of objects in a higher degree of detail than the eye of an insect in fact discerns.

The images reproduced by the means described here are shown in Figs. 2–6. Before proceeding to analyse them I should recall that these photographs can only give an indication of the amount of information provided by the compound eye, i.e. of its power of discrimination between one detail and another. These photographs do not therefore in any sense illustrate the optical distortions of the retinal image which may occur in the eye [6], and which have no general relationship to the objective depiction by the eye of elements of similarity and difference in the outside world. This is associated with the fact that in insects, as in higher forms of animal life, not only the eyes (the photoreceptors) but also specific parts of the central nervous system take part in visual perception. This concept is well illustrated by an example connected

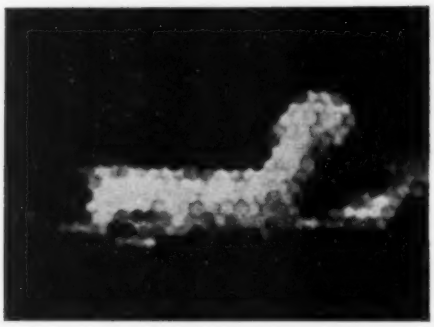
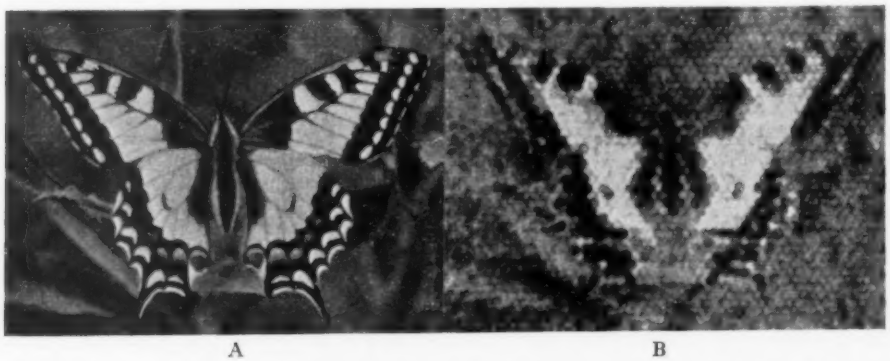


FIG. 3. The image of a Privet Hawk Moth caterpillar with the detail perceived by a bee from a distance of 20 cm.

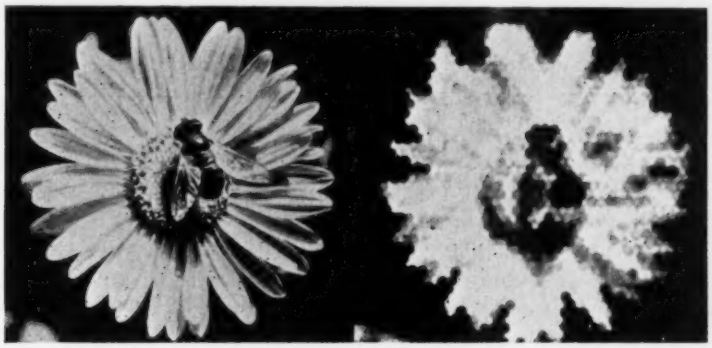
with the functioning of the eyes of vertebrates and of man. We know that the crystalline lens creates an inverted image of objects on the retina, but nevertheless we see them in their true alignment. In consequence, the photographs given here can only illustrate what the insect's eye sees (distinguishes), and give no information as to how the insect senses any object.

Fig. 2 (B) shows the image of a privet hawk moth caterpillar (*Sphinx ligustri* L.) with the detail distinguished by the eyes of insects. Over its length this image covers 45 grid openings. For the eyes of insects such as the honey bee or the *Aeshna* genus dragon-fly, the angles between ommatidia axes of which are roughly 1°, this photograph of a caterpillar reproduces the detail distinguished by an eye when actually seeing at a distance of 9·5 cm. For the eyes of a *Zygaena* genus butterfly, whose angular ommatidium density is half as much, the same photograph will correspond to the depiction provided by a compound eye regarding an object from half that distance, i.e. from a distance at which the angular dimensions of the caterpillar reach 90°. The photograph reproduces the details of the pattern and shape of the caterpillar quite well, particularly if examined from a distance of 1·5–2 m. The transverse bright and dark lines on the body of the caterpillar are observable, but the boundaries between abdomen segments and the detail of head and legs are not distinguishable.



A B

FIG. 4. A—The Swallowtail butterfly (*Papilio Machaon L.*). B—The image of a Swallowtail showing the detail seen by a bee of the butterfly's wings from a distance of 10 cm.



A B

FIG. 5. A—The *Eristalis* fly on a flower. B—The image of the same objects, giving the detail distinguished by a bee from a distance of 9 cm.

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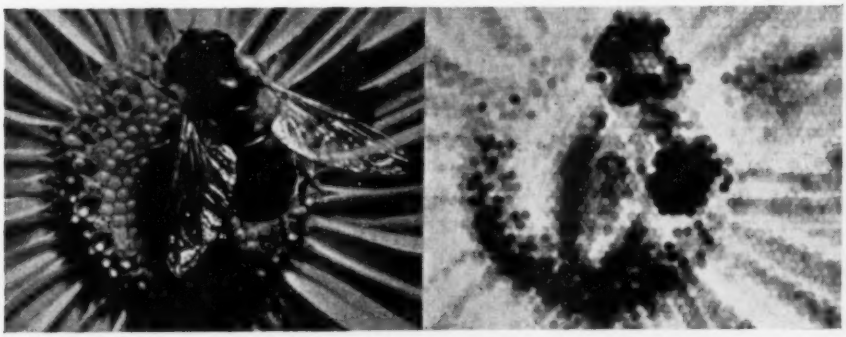
Fig. 3 illustrates the image of the same caterpillar in the amount of detail seen by a *Zygaena* genus butterfly from a distance of 10 cm or by a bee or dragon-fly from a distance of 20 cm. The reproduction of detail is considerably worse than in the previous photograph.

The image of the swallowtail (*Papilio machaon* L., Fig. 4 (B)) covers 45 grid channels in width; it provides the detail which a bee perceives on the butterfly's wings from a range of 10 cm. The general picture of the wings is quite distinctly seen, though their individual details have not been resolved—they are shown as spots whose clarity is somewhat less than the basic light field of the wing.

The image of the *Eristalis* fly on the flower in Fig. 5 (B) covers 10 grid cells or 10 ommatidia, equivalent to the vision of a bee in natural conditions from a distance of 9 cm. Part of the same photograph is given in Fig. 6 (B), showing considerably greater detail; this is for a case where a bee is seeing the fly from a distance of 3·5 cm, i.e. at an angle of vision of 24°.

These photographs indicate the amount of information given by the eye for those cases alone where the angular density of the ommatidia is identical for every diameter



A B

FIG. 6. A—Detail of Fig. 5. B—The image of fly and flower, giving the detail distinguished by a bee from a distance of 3·5 cm.

of the eye. We know, however, that the angular density of the ommatidia and the visual acuity of certain insects, for instance bees, are not the same in different portions of the eye.

Analysis of Figs. 2–6 enables us to state that the apposition eye makes it possible to distinguish quite small details in the living objects with which insects are concerned in natural conditions. The vision of the apposition eye may be even more acute when we consider moving objects. All this is only correct, however, for cases where insects are viewing objects at close ranges of the order of a few centimetres. The compound eye is not capable of distinguishing details of objects seen at relatively great distances. Groups of large but very distant objects are also beyond the limits of resolution by the eye in view of their small angular dimensions compared with the angles between the optical axes of the ommatidia.

SUMMARY

A photographic method of reproducing the images of objects with the amount of detail provided by the apposition eye of insects is described. Photographs of a number of living objects have been taken by this method, giving the detail which the eyes of various types of insect can resolve when viewing their natural surroundings at different distances. The acuity of insect's vision is calculated according to the number of ommatidia embraced by one and the same angle (the angular density of the ommatidia) and not from the insect's angle of vision, the method used by a number of authors.

Translated by J. H. DIXON

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THE BIOELECTRICAL PROCEDURE FOR STUDYING REDUCTION IN THE SENSITIVITY OF THE AUDITORY ANALYZER UNDER THE EFFECT OF POWERFUL SOUND STIMULI*

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NUMEROUS research projects devoted to the problem of the reduction in the sensitivity of the auditory analyzer related to the intensity, duration, pitch and nature of a stimulating sound have been based on the use of the subjects' verbal response reaction. However, use of the verbal report for the delicate analysis of the relationship

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between the nature of an external sound stimulus and the state of the central nucleus of the auditory analyzer is not possible, since there is no way of measuring the degree of perception.

This article concerns the problem of the electrocorticographic study of reduction in the sensitivity of the auditory analyzer of cats with relation to the duration of an adequate stimulus, performed by analyzing the retardation in primary electrical reaction after the application of very powerful pure tones. The value of performing this task using pure tones could be gauged when recording the biopotentials from the relevant afferent sensory tracts of the cortex.

The great changeability of the structure of the various architectonic fields of the brain cortex and the structural variableness of the cells of the various segments of the central nervous system are probably very closely associated with the physicochemical changes which arise in the corresponding structural developments under the action of adequate external agents.

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If we regard the nerve process which develops in the grey matter centres under the action of an external agent not as being of a fixed established nature, but rather as being at various points on their evolutionary path, we can assert that the physicochemical changes caused by action from the external environment precede structural change. The physicochemical change caused in a cell by a stimulus is at the basis of (i.e. is the first stage of) the subsequent and simpler structural change. The higher the energy of a stimulus and the longer its duration, and consequently the greater the extent to which the physicochemical dynamic equilibrium of an affected cell is upset, the higher the residual phenomena must be and the longer it must take to revert to its original level. Upset dynamic equilibrium of this nature does not return to normal immediately on removal of the cause, but does so over a definite period, leaving behind it a certain residual effect.

Since different frequencies cause different residual effects, which are also at the basis of stable structural change, they lead to the inevitable formation of corresponding tone centres.

The works by Danilevskii [1], Larionov [2], Sarkisov and Livanov [3], Sarkisov [4], Gershuni [5], Artem'ev [6], Kogan [7], Bremer [8], Tunturi [9], Thomas [10], Schuknecht [11], Hind [12], Mickl and Ades [13], Hind and Schuknecht [14], Roitbak [15] and others were directed at the problem of the localization, in the temporal segments, of the individual tone centres by recording bioelectrical response reactions.

Tunturi [9], Hind [12] and Hind and Schuknecht [14] established, by the oscillographic recording of primary bioelectrical responses, that in dogs and cats high tones are localized in the front part of gyrus suprasylvius anterior, low tones in the upper part of gyrus ectosylvius anterior and the upper third of gyrus ectosylvius posterior (Fig. 1). The research was carried out by recording the primary bioelectrical reactions of narcotized animals to the effect of a specific tone of threshold intensity after the local application of a 3% solution of strychnine.

My experiments were made on narcotized cats, biopotentials being recorded directly from the cortex surface. The high-frequency range (4000 c/s) was investigated. A 1% solution of sodium amytal was used as narcotic. The electrodes were silver wires of diameter 0·3 mm. The bioelectric potentials were recorded at two

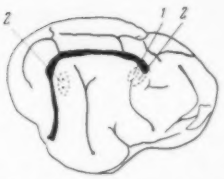

Fig. 1. The localization of the high frequency (1) and low frequency (2) afferent sensory tracts in the large hemisphere cortex of a cat. These were investigated by the method of registering action potentials after the application of strychnine (Hind and Schuknecht). The regions in which the highest amplitude potentials are set up are indicated by dots.

poles, the distance between electrodes being 2 mm. The biocurrent was recorded on a two-channel cathode-ray encephalograph. A type 3G-10 sound generator was used as stimulation source. The intensity of the sound stimulus was measured by a voltmeter at the output side of the sound generator. Voltage steadiness in the telephone during the experiment was also controlled by this voltmeter.

By 1–1·5 hr after administering 50 mg of narcotic per kg of body weight, the temporal muscle, the temporal bone and the dura mater were ablated. The biocurrents were recorded from the sectors indicated in Fig. 1.

After 1·5–2 hr of narcosis, considerable bioelectrical activity was observed in the auditory area of the cortex. A regular α-rhythm of frequency 13–18 c/s (Fig. 2(A)) was observed to appear gradually as narcosis was deepened by further administration of sodium amytal. The application of a sound stimulus in the background of this activity caused reduction in the amplitude of the α-rhythm and reduction in the frequency. Further reduction in the rhythm (Fig. 2(B)) and finally complete absence of cortex bioelectrical activity (Fig. 2(C)) were observed as narcosis was deepened.

After checking that there was a complete absence of background electrical activity

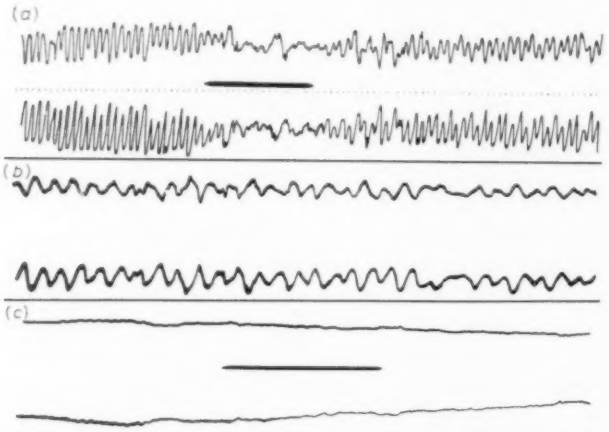

Fig. 2. Checking of the background bioelectrical reaction of the temporal area cortex as narcosis (sodium amytal) is deepened. A—the effect of a sodium amytal dosage of 50–60 mg per kg weight. The α-rhythm is 17 c/s. B—the effect of a sodium amytal dosage of 70–80 mg per kg weight. Slow waves: 6–8 oscillations/sec. C—the effect of a dosage of 100–110 mg of sodium amytal per kg weight. No background activity.

(following the administration of 70–80 mg of narcotic per kg of body weight), cortical response to weak sound stimuli was observed to be absent. Moistening the recording electrode tips with a 1% solution of strychnine and their application to the area under investigation caused strychnine spikes. Where the electrodes were in the appropriate afferent sensory tract (4000 c/s), the sound stimulus caused a regular response for each application in the form of a two-phase initial effect. Stronger sound stimuli of 95–100 dB (300–500 mV) gave rise to a generalized response over the entire auditory analyzer.

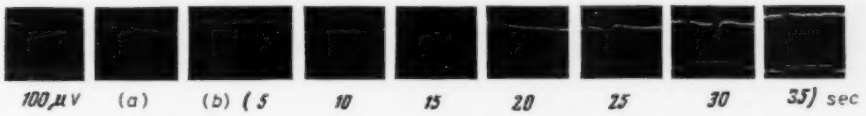

FIG. 3. Restoration of the amplitude of the action potentials recorded from the cortex (h.f. region) after the application for 10 min of a pure 4000 c/s tone of intensity 117 dB. A—the bioelectrical action potential before applying the powerful sound. B—the bioelectrical action potentials after cessation of the powerful sound.

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I tried deepening the narcosis by raising the dose of sodium amytal to 100–110 mg per kg of body weight and repeating the same experiments without the strychnine. These experiments were successful. Where the electrodes were applied in the appropriate afferent sensory tract, we regularly received an initial positive effect for each 4000 c/s sound stimulus. It was characteristic that an area of about 1–1·5 mm², from which the positive action potentials of highest magnitude are recorded for an external pure tone of 4000 c/s and the given strength, was found in the area indicated. When the electrodes are gradually moved 2–3 mm away, the amplitude of the potential falls until there is no reaction at all to sound of that strength. Interchanging the recording electrodes was attended by reversal of the direction of the reaction. Low-frequency sound (400–800 c/s) caused no reaction.

Increase in the sound stimulus intensity led to increase in the amplitude of the initial response reaction and to increase in the area around the maximum reaction focal point from 2–3 mm for a stimulus strength of 89–91 dB (150–200 mV) to 5–6 mm for a stimulus strength of 104–105 dB (800–1000 mV).

Further increase in stimulus intensity did not alter the amplitude of the initial positive effect. These data of mine concord with the results obtained by Mickl and Ades [13] and Roitbak [15]. The phenomenon of increase in positive potential amplitude with increase in sound stimulus intensity is explained by Gershuni [16], with whom Never and Broy [17], Adrian [18] and Derbyshire and Davis [19] agree, as due to implicating a greater number of sound receptors when the sound oscillation intensity is increased. Golembos and Davis [20], and also Tasaki [21] explain this phenomenon by an increase in the number of impulses in separate fibres of the auditory nerve when the intensity of the sound stimulus is increased. Roitbak [15] quotes a great number of facts as evidence supporting the idea that the initial positive potential reflects the activity of the grey matter neurons. Gershuni [16], Bremer [8, 22], Artem'ev [6], Chang [23] and others support this opinion. Smirnov [24] shows, on the basis of the results of his work, that it is probably correct to adopt the action potentials as indicators of the functional state of the brain cortex.

My data and those of other authors on study of the changeability of the initial bioelectrical responses, and on the localization of the different tones in the cortex, enable us to use this change in action potentials to study changeability in the sensitivity of the neurons in the various afferent sensory tracts of the cortex with relation to the intensity, duration and frequency of a sound stimulus.

Some data from my study of reduction in the sensitivity of the grey matter nuclei of the auditory analyzer of a cat after the application of a pure tone (4000 c/s) of intensity 117 dB for 10 and 30 min are given below.

The procedure for my experiments was as follows. After deepening narcosis until background bioelectrical activity was non-existent (100 mg of sodium amytal per kg of weight of the cat), the focal point of maximum amplitude for initial positive response to the effect of a pure tone (4000 c/s) of intensity 89 dB (150 mV) was investigated. After an absence of reaction to a low-frequency tone (400–800 c/s) of the same intensity had been observed, and several identical maximum readings had been obtained in succession for the effect of a high frequency tone (4000 c/s), a sound signal of intensity 117 dB was transmitted to the animal's ear. We then recorded the time interval after cessation of the sound signal during which the initial potential amplitude for a pure sound of intensity 89 dB was regained. Two type 3G-10 sound generators were used as the source of stimulation. One of them generated the powerful sound (117 dB): the other was used for investigating the extent to which sensitivity had been reduced by transmitting a sound signal into the same telephone after cessation of the powerful tone (sound indicator intensity 89 dB). The degree of intensity of the signal was controlled by an attenuator. The durations of operation of the powerful sound were 10 and 30 min. The control sound signal (the indicator) was transmitted in separate consecutive pulses after 1, 3, 5, 10, 15, 20, etc., sec.

Fig. 3(A) shows the change in the potential, recorded at the maximum amplitude focus, for the sound stimulus of 150 mV (89 dB), which was used as the indicator for observing the functional displacements in the cortex caused by a powerful sound. The same illustration shows the changes in cortex positive potential under the effect of separate consecutive sound pulses of the same strength transmitted 5, 10, 15, 20, 25, 30, 35 sec after the application of an intense tone of frequency 4000 c/s, strength 117 dB for 10 min. We can see from the recording that the minimum cortical response reaction is observed only at the tenth second after cessation of the powerful sound; the response reaction intensifies in proportion to the time which has elapsed since the cessation of this sound, and is fully restored in 35 sec.

The time taken to restore the sensitivity of a cortex, however, which has reached a state of check under the action of a stimulus of specified intensity and frequency, alters considerably from animal to animal (from 3–10 sec to 3–4 min) in spite of uniform times of application of the stimulating agent and strictly measured narcotic doses (100 mg per body kg). This variability in time taken by the restoration of sensitivity can be explained by type-differences in the features of different animals. The time taken for the cortex to regain sensitivity after a powerful tone of duration 30 min is of a more lingering nature. The nature of the restoration of sensitivity in the cortex after a lengthy stimulation (30 min) is distinguished both by increase in the length of the restoration period and by non-uniformity of this period.

FIG. 4. The restoration of the amplitude of the action potentials recorded from the cortex (h.f. region) after the application for 30 min of a pure 4000 c/s tone of intensity 117 dB. A—the bioelectrical action potential before applying the powerful sound. B—the bioelectrical action potentials after cessation of the powerful sound.

Fig. 4 illustrates the course of restoration of sensitivity in the cortex after the application of a powerful sound for 30 min. Restoration of the maximum amplitude of variation in potential for the 89 dB sound ensued in the twenty-eighth minute after cessation of the intense sound.

I have described above my experiments in studying the degree to which the sensitivity of the cortex is reduced, in which I used the method of measuring the rates at which it was restored. In order to determine the degree by which cortical sensitivity was reduced by the impact of an intense sound stimulus, I also used measurements of the difference in intensity levels of the indicators used before and after the application of the powerful sound for which these cause variations in potential of equal amplitude.

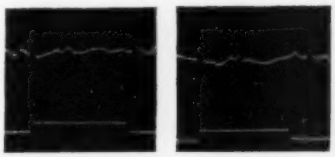

FIG. 5. Identical amplitudes of variation in potential for a sound of intensity 89 dB prior to application of the powerful sound (A) and 94 dB after cessation of the powerful sound (B).

I performed these experiments as follows: the amplitude of the variation in positive potential for a sound of intensity 89 dB was determined prior to the application of the powerful sound. The amplitude of the response reaction to an indicator exceeding the initial indicator in intensity was measured after a 4000 c/s tone of intensity 117 dB had been transmitted for 10 and 30 min. The use of an indicator of higher intensity after the cessation of the powerful sound revealed a response reaction whose amplitude increased, the higher the intensity of the indicator. By feeding a higher voltage to the telephone (regulating by the attenuator) immediately after the cessation of the powerful sound, I was able to select a strength which caused a cortical response reaction, the amplitude of variation in the potential of which was equal to the amplitude of variation in potential for the initial indicator (89 dB) prior to transmission of the powerful stimulating agent.

By determining the difference in intensities, for equal amplitude of variations in potential prior to and after the use of intense sound, the degree by which the sensitivity of the cortex had dropped could be judged. The amplitude of the initial positive reaction of the cortex to 89 dB sound was 100 μV. The same amplitude was obtained

when using sound of intensity 94 dB immediately after the cessation of the powerful stimulus. The difference in displacement is 5 dB.

A similar phenomenon was observed when applying the powerful sound for 30 min. The difference in intensities increased when powerful sound stimulation was applied for the longer period (30 min). However, this difference in indicator intensities differs considerably from animal to animal.

SUMMARY

(1) The phenomenon of reduction in the amplitude of the cortical action potentials after the application of a powerful adequate stimulus may serve as an objective indication of the degree by which cortical sensitivity has dropped. Increase in the length of time for which the powerful sound stimulus is applied is accompanied by an increase in the period necessary to restore the initial amplitude of the action potential.

(2) The time taken to regain the initial amplitude of the action potential, after the application of powerful sound, changes with the intensity and duration of the sound. It can be used as an index of the degree of reduction in the sensitivity of the auditory analyzer.

(3) The different intensities of the sound stimulus used before and after transmission of the powerful sound, intensities for which identical amplitudes of the action potential can be obtained, may also be used as an indication of the degree of reduction in the sensitivity of the auditory analyzer.

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ALTERATION IN THE ADAPTATION OF THE HEARING TO SILENCE WITH RELATION TO THE DURATION OF A PRELIMINARY LOUD NOISE*

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THE duration of a sound stimulus is of great material importance in the effect of such stimuli of high intensity on a human being [1]. We know from the works of a number of authors [2–4] that the rise in the audibility threshold after an intense sound stimulus is greater if the duration of the stimulus is increased. In conformity with this, if the stimulus has been more prolonged, the final restoration of the audibility threshold to its characteristic level for quiet conditions, at which it stood before stimulation, takes longer. Cases are known where the complete restoration of sensitivity has taken several days.

At the same time it has been established by a number of investigations [5–9] that the process of alteration in the level of sensitivity in the auditory analyzer with relation to changes in acoustic environment, i.e. the process of adaptation of the hearing, takes place extremely rapidly. It has been proved that the restoration of subjective loudness is completed within a fraction of a minute, while the restoration of the audibility threshold takes several minutes. In this connexion, cases where the return of the audibility threshold to normal is prolonged are indicators of disruption of the normal course of the adaptive process; this is considered to be a manifestation of the fatiguing effect of a sound stimulus.

The object of this article is to trace the course of the development of disruption in the adaptive capacity of the auditory analyzer with relation to the duration of a sound stimulus.

The importance of research in this field is determined by the fact that the prolonged action on human beings of intense sounds (noises) is very widespread in industry, while the mechanism whereby they exert their effect, and equally the normal times for which people may remain and work in such conditions, has not yet been sufficiently studied.

We observed changes in the course of the subsequent adaptation of the hearing to quiet on increasing the duration of stimuli from 0·5 to 180 min. The research was carried out on students, men 20–25 years old, who had previously been examined by neuropathologists and otolaryngologists. Persons passed as fit were selected for the tests.

Noise with a uniform frequency characteristic and ultimate intensity of 117 dB was used as the sound stimulus. Fig. 1 is an illustration of the spectrum characteristic

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of the noise, recorded in the laboratory by engineer V. P. Babkin. Tones of 200, 2000 and 4500 c/s were used as the sound signals for determination of sensitivity of hearing, these representing the low, middle and high regions of the audio-frequency range. The tests were performed under laboratory conditions using the installation whose layout is illustrated in Fig. 2.

The noise was produced by magnetophone and passed through an amplifier to a type TD-6 electrodynamic telephone which served as noise-radiator. The sound signal in the form of a pure tone was transmitted to the same telephone. The source for these tones was a sound-frequency generator. An attenuator included in the circuit enabled us to transmit measured quantities of the sound signal (i.e. the tone) to the subject.

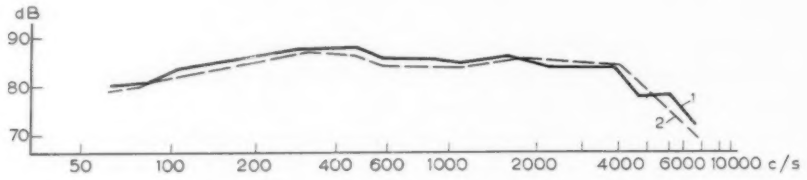

FIG. 1. Spectrum characteristic of the noise. Abscissa—frequency; ordinate—degree of intensity; 1—telephone No. 2A, 2—telephone No. 21.

The person performing the tests measured the thresholds of audibility by gradually increasing the sound signal from inaudible to audible. The subject signalled the commencement of audibility by pressing a button which switched on a pilot lamp in front of the experimenter. Subjects were instructed not to report perception of the sound until they could distinguish its tonality. An audiograph recording was made, prior to beginning the tests, for each subject, i.e. the sensitivity of his hearing was determined over the whole audio-frequency range. A single tone only was used as the tonal signal in each test. Each test began with measurements of the thresholds of audibility in silent conditions for the specified tone. Measurement of the thresholds

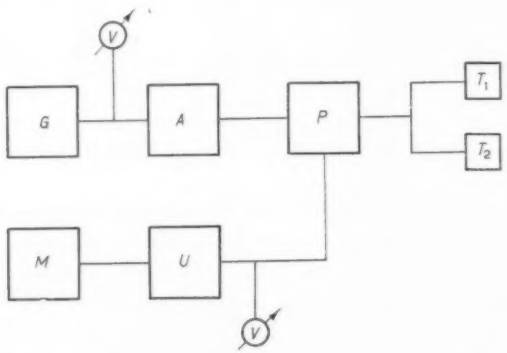

FIG. 2. Diagram of the installation for measuring audibility thresholds and producing noise. *G*—3G-2A generator; *A*—attenuator; *P*—switchgear; *M*—MAG-800 magnetophone; *U*—amplifier; T_1 and T_2—DT-6 telephones; *V*—voltmeter.

continued for 10–15 min at the rate of 4–5 times a minute, while the measurement cycles were staggered irregularly with rest periods, in order to exclude the production of time reflexes in the subjects. The measurements indicated enabled us to determine a mean value for the audibility thresholds of the subjects in comparative silence and the limits between which the thresholds ordinarily fluctuated. The noise was then switched on; it was left on for different lengths of time in the various tests, 12 different variants of duration being used between 0·5 and 180 min. In one group of tests the audibility thresholds were only measured before and after the application of the noise. In another group of tests the sensitivity of hearing was determined immediately the noise had been switched on, with the sound muffled. Under these

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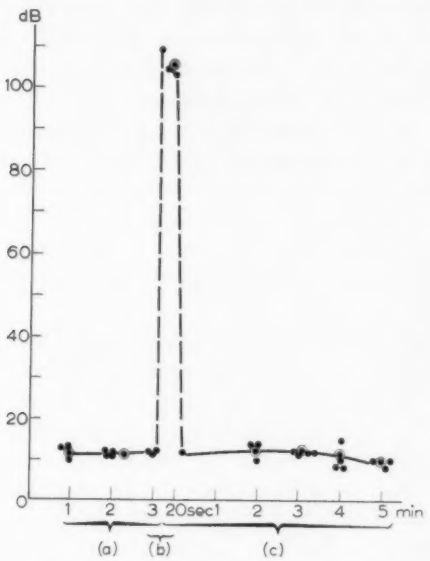

FIG. 3. Typical curve for noise of 0·5 min duration. Abscissa—time; ordinate—degree of intensity. Threshold values: *a*—before noise; *b*—during noise; *c*—after noise. Subject Kh. Sound signal 2000 c/s.

conditions the thresholds were measured several times during the first 2–3 min during which the muffling noise was operating. Further measurements were made with the object of finding out the course of changes in the auditory analyzer connected with cessation of the noise. For this purpose, the audibility thresholds for the relevant tone were measured immediately before the noise was switched off. The noise was then switched off and measurements of the threshold for the same tone began at once in silent conditions.

With my procedure for measurement, the value of the audibility threshold, in a number of cases, could not be determined before 20 sec had elapsed after switching off the noise. So the measurement taken 20 sec after the noise was switched off was

taken as the basic value characterizing the sensitivity of the hearing after stimulation in all the tests. The course of the restoration of sensitivity of hearing in silent conditions was as a rule systematically observed for 20–30 min, and in a number of separate tests for up to 90 min, after the noise had been switched off.

Mean threshold values measured before the noise was switched on were compared with the threshold values taken while the noise was on to determine the value signifying the adaptation of hearing from silence to noise. In order to observe the relationship between audibility threshold values during muffling and the length of time during which the noise was on, threshold values measured at the beginning and end of the muffling action by noise in each test were also compared with one another.

The audibility threshold value measured 20 sec after the noise had been switched off was further compared with the threshold value taken prior to stimulation, this enabling us to establish the displacement of the audibility threshold; the threshold value taken 20 sec after the noise had been switched off was in addition compared with the value measured during muffling by noise. This latter comparison showed the magnitude (expressed in dB) by which the sensitivity of the hearing rose in the 20 sec elapsing after the noise was switched off; henceforth I shall call the difference in threshold values thus obtained values for adaptation to silence. The relationship was then established of the displacement of the audibility threshold, and of the value of the adaptation to silence, to the length of time for which the noise had been on and the frequency of the tone being used. The work was based on the results of 286 tests performed on nine subjects.

In these tests the audibility threshold did not exceed its initial value, where the noise was switched on for not more than 0·5 min, in measurements made 20 sec after the noise was cut off. The results of a typical test on subject Kh. are given in Fig. 3. The audibility threshold value in silent conditions prior to switching on the noise fluctuated between 10 and 12 dB for this subject, the mean value being 11 dB.

When the muffling noise is switched on, the threshold of frequency for a given tone, as we know, exceeds the threshold in silent conditions by various amounts depending on a number of factors—the intensity and spectrum characteristic of the noise, and the individual peculiarities and functional state of the subject. This change in threshold value expresses itself in the adaptation of the hearing to the perception of sound signals during intense sound interference.

In the experiment indicated, the audibility threshold measured 25 sec after the noise had been switched on was 109 dB, i.e. it exceeded the mean initial silent conditions threshold value by 98 dB. This difference between the threshold values taken before and during the noise effect we will call the value for adaptation to noise. Twenty seconds after the noise had been switched off the audibility threshold value was already within the limits between which it had fluctuated prior to stimulation. During this time the threshold had dropped by 92 dB, this being an expression of the full adaptation to silence of the hearing, since the threshold had reached its initial level. Thus, in this test adaptation to silence was very quickly accomplished. As concerns adaptation to noise, to judge its final value it was necessary to trace the changes in the threshold as the length of time for which the noise was on was increased.

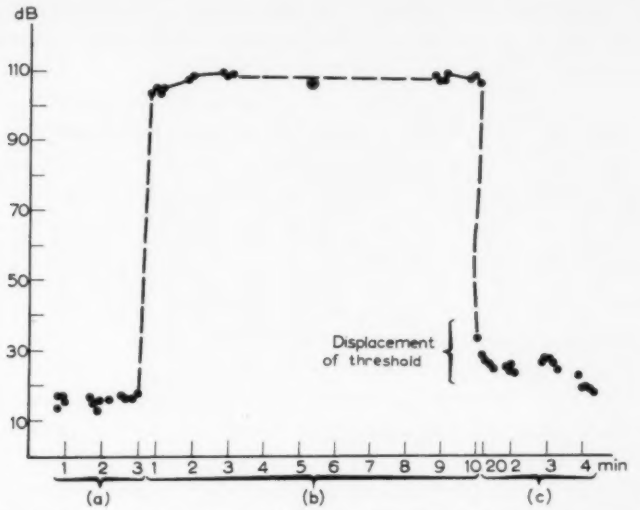

FIG. 4. Typical curve for noise of 10 min duration. Abscissa—time; ordinate—degree of intensity. Threshold values: *a*—before noise; *b*—during noise; *c*—after noise. Subject Kh. Sound signal 2000 c/s.

Fig. 4 illustrates the results of a typical test with the noise on for 10 min. In measurements made 20–30 sec after the commencement of muffling by noise, the audibility threshold was 102 dB. The threshold did not change during repeated measurements made over 9·5–10 min. Evidently adaptation to noise was already complete when the threshold was first measured. But 20 sec after the noise was switched off, the audibility threshold still considerably exceeded its initial value (by 13 dB). In consequence, in the same lengths of time, the sensitivity of the hearing was restored fully after the noise had been on for 0·5 min, and only partially after it had been on for 10 min.

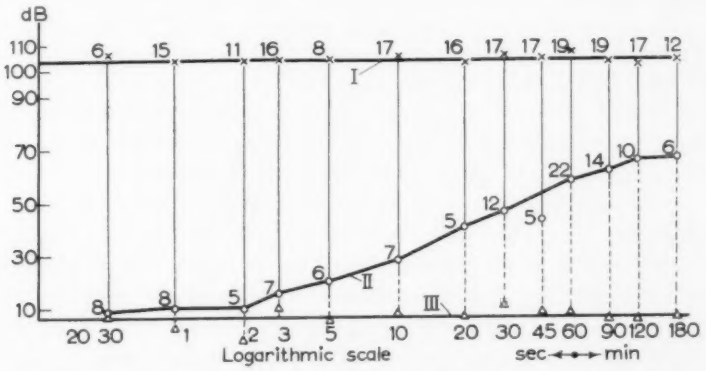

FIG. 5. Composite experimental data for different noise durations, for the whole group of subjects. Abscissa—logarithmic time scale. Average audibility threshold values: I—during noise; II—after noise; III—in silence before noise. Sound signal 2000 c/s. The figures give the number of tests.

Comparison of the results of these tests enables us to assume that this latter circumstance is the result of alteration in the process of adaptation of the hearing to silence under the influence of more prolonged noise. There are, however, works of greater scope [10, 11] which confirm the correctness of this assumption, since they prove that the audibility thresholds for various tones perceived against a background of noise are always very rapidly established at a certain level and do not alter thereafter with relation to the duration of stimulation, for durations of up to 3 hr [10]. This is also confirmed by the data from my work given in Fig. 5.

In Fig. 5 a line parallel to the abscissa is drawn at the top at the level of the average value of the audibility threshold during the application of noise. The differences between the average values of the thresholds measured at various times during the application of noise represent a scattering of indices which we can see is not dependent on time. The vertical lines connecting the abscissa to the line parallel to and above it, drawn in unbroken and dotted sections, show the amount by which the audibility threshold of a group of people has altered in comparison with their thresholds prior to the use of noise. It is apparent from the illustration that these values, which I called the values of adaptation to noise, remain identical for different moments during the transmission of noise.

One might therefore think that the changes in the auditory analyzer, which are associated with the commencement of intense noise and represent the adaptation of the hearing to the specific acoustic effect, are very rapidly completed, i.e. in the first minute, and do not depend on further continuation of the noise.

On the one hand, the data furnished permit one to suppose that the raised audibility threshold observed in silent conditions after the noise is not associated with changes or disturbances in the process of adaptation of the hearing to noise. On the other hand it follows, from the fact that the value of the audibility threshold with noise in operation is very rapidly established and does not alter with time, that the adaptation of the hearing to silent conditions must commence from about the same level, whatever has been the duration of the noise preceding the silence.

We saw from Figs. 3 and 4 that the displacement of the audibility threshold 20 sec after the cessation of noise differed greatly for noise transmission times of 0·5 and 10 min: in the first case adaptation to silence was complete, in the second it exceeded the initial threshold value by 13 dB. The audibility thresholds measured immediately prior to switching off the noise were practically identical in both cases (102 and 103 dB); therefore the disparity in threshold values, for the same times after the cessation of noise, indicated above, could only be the result of different rates of restoration of hearing in the 20 sec. Evidently, full adaptation of the hearing to silence did not occur in the case of 10 min of noise, and a displacement was observed in the threshold in comparison with its initial level in silent conditions, because the rate of restoration of the hearing after 10 min was less than that after 0·5 min.

The curve in Fig. 5 shows the relationship between the mean values for the audibility threshold taken 20 sec after the cessation of noise and the duration of the noise in a whole group of tests. The sections of the vertical lines connecting points on the straight line parallel to the abscissa to the corresponding points on the curve show the values in dB to which the threshold had fallen 20 sec after the cessation of noise, or represent the value which I have called the value of adaptation

to silence. The dotted sections of the vertical lines connecting points on the curve to the corresponding points on the abscissa show the displacement of the threshold in 20 sec after the cessation of noise in comparison with the threshold in silence prior to stimulation. We can see from Fig. 5 that there is no displacement of the threshold if the value of adaptation is high, and that the lower the adaptation the greater the amount by which the threshold is displaced. Thus, we may assume that displacement in the audibility threshold after the cessation of noise is actually the result of deceleration of the process of adaptation of the hearing to silence. We can trace the course of this process with relation to the duration of operation of the noise (Fig. 5). We see that the value for adaptation falls, and the displacement of the threshold rises proportionately, with increase in the duration of the noise. However, careful analysis of the picture shows that there is no direct relationship between the alteration in both the values mentioned and the increased duration of stimulation. The rate of reduction in the value of adaptation to silence, and the corresponding rate of increase in the displacement of the threshold, slow down in proportion to the rate of increase in the duration of the preceding noise.

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Fig. 6 shows curves for the relationship between displacement in the audibility threshold and the duration of noise. As distinct from the data given in Fig. 5, displacement in the threshold was here brought out by the comparison of the corresponding values for each test individually and by subsequent averaging of the displacements found.

We can trace, in Fig. 6, the way in which displacement of the threshold changes, with alteration in the duration of stimulation, by the same amount in the various groups of tests. A table for the mean data corresponding to curve 2 in Fig. 6 is given below (Table 1).

Analysis of the graph and Table 1 shows that increase in the duration of noise to identical values provides identical effects in the various groups of tests. Where the noise duration was 0·5 min, the displacement of the threshold was 2 dB. Increase in the duration of stimulation to 30 min leads to an increase in displacement of 33 dB. If, however, the duration of this last group of tests is increased by a further 30 min the displacement rises less (by 16 dB). Tests in which the duration of stimulation is 90 min will be distinguishable from the group of tests in which the noise duration was 60 min by a still lower increase in displacement. Beyond this the increase in displacement becomes so slow that even when the duration of noise is increased by

Table 1. Average displacement of threshold with change of duration of stimulation

Duration of noise (min)	Displacement of threshold with relation to its level before stimulation (dB)	Difference in amounts of displacement by comparison with the preceding group of tests (dB)
0·5	2	—
30	35	33
60	51	16
90	56	5
120	61	5
180	61	0

60 min (from 120 to 180 min) practically no difference in the amount of displacement can be observed.

The regularity of displacement in the threshold which is observed represents in essentials the regularity of reduction in the capacity of the auditory analyzer to adapt itself to silent conditions relative to increase in the duration of the preceding noise. The data given prove that this regularity follows an exponential curve.

We may assume that reduction in the adaptive capacity of the auditory analyzer resulting from increase in the time for which a man has been exposed to the effect of intense noise is the result of the fatiguing effect of that noise. Quoting the opinions of a number of authors, Tanner [12] gives several features which distinguish the

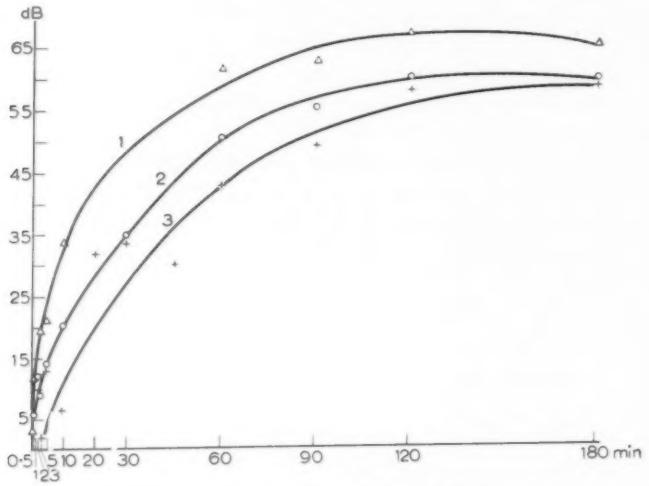

FIG. 6. Relationship between the displacement of the audibility threshold and the duration of noise. Abscissa—duration of noise; ordinate—displacement of the threshold. Curves: 1—for subject K; 2—mean data for the whole group of subjects; 3—for subject Kh. Sound signal 2000 c/s.

phenomenon of fatigue in the auditory analyzer from the process of adaptation. He points out that fatigue displays considerable dependence on the duration of aural stimulation, while adaptation is practically independent of this duration.

From this point of view it should be stated that the laws which I have specified are exclusively concerned with fatigue of the auditory analyzer. However, it is more correct to consider that we have been observing changes in the adaptive process under the influence of the phenomenon of fatigue.

I consider that it is possible, without excluding the importance of the effects of noise on every part of the auditory analyzer, to draw attention to the probability that it affects the central part of the auditory analyzer (on the basis of the results of a number of investigations).

Data exist [13] which indicate changes in the adaptation process occurring under the influence of hypnosis, i.e. under the influence of alteration in the state of the nerve processes in the central nervous system: changes in the human hearing functions

after the action of a tonic stimulus of 1000 c/s at a strength of 100 dB have been studied. In cases where it was suggested to the subject, during stimulation, that he was in a silent place, increase in the thresholds after stimulation was considerably less than in experiments without hypnosis. It is interesting that when it was suggested to a hypnotised subject that he was hearing gunshots his audibility thresholds after the seance had increased.

When the organ of hearing was stimulated by a loud noise (80–100 dB) for 5 min during hypnotic sleep, the noise did not cause great changes in the audibility threshold; in this case the thresholds were little different from those in tests in which hypnotic sleep was not accompanied by sound stimuli [14]. All these data indicate that the central nervous system plays a part in controlling the adaptive process in the analyzer.

I observed in my previous aritcle [15] that in cases where subjects were given a moderate dose of phenamine 1 hr before they were subjected to noise, their audibility thresholds 20 sec after cessation of the noise were as a rule considerably lower than in tests where phenamine had not been administered. We know phenamine to be a preparation which stimulates the central nervous system, so I assumed, in accordance with Fadeeva's data and remarks [16], that this effect of the use of phenamine was governed by increase in the activity of the nerve processes stimulated by it. It is extremely probable that the adaptive capacity of the auditory analyzer increased owing to an equivalent effect of the preparation and that a more profound regaining of the level of aural sensitivity took place in the first moments after the cessation of noise. The fact that the adaptive capacity of the auditory analyzer drops at a constant rate in proportion to increase in the duration of noise indicates, in its turn, the existence of an adaptability mechanism which ensures the possibility of regaining the functions of hearing after a very prolonged intense noise, even though this capacity deteriorates.

As was noted above, the reduction in the audibility threshold, during the 20 sec after the cessation of noise, to the value required to regain the initial audibility threshold only took place in cases where the noise was on for not more than 0·5 min. In every other case the threshold for that particular moment dropped to a lower value relative to the level characteristic of the threshold of my subjects in conditions of muffling by the given noise; therefore by 20 sec after the cessation of noise it exceeded the initial threshold by a certain amount.

It was shown above that restoration of the hearing commences, for a specified stimulation characteristic, from a roughly constant mean level corresponding to the audibility threshold of the given group of subjects during noise; nevertheless, 20 sec after the moment of cessation of noise the audibility thresholds differ, depending on the duration of the noise; this is evidently the result of different rates of adaptation during these 20 sec. This is shown in schematic form by the dotted lines on the left-hand side of Fig. 7. The unbroken lines in Fig. 7 show the restoration in hearing in a period following 20 sec after the noise was switched off. In view of what has been stated above, restoration began from different levels in the various groups of tests. However, notwithstanding this, the fact that the curves for restoration of the thresholds after different durations of noise thereafter follow parallel paths invites our attention. This circumstance indicates that at a later period the rate of restoration of the threshold ceases to be dependent on the duration of stimulation.

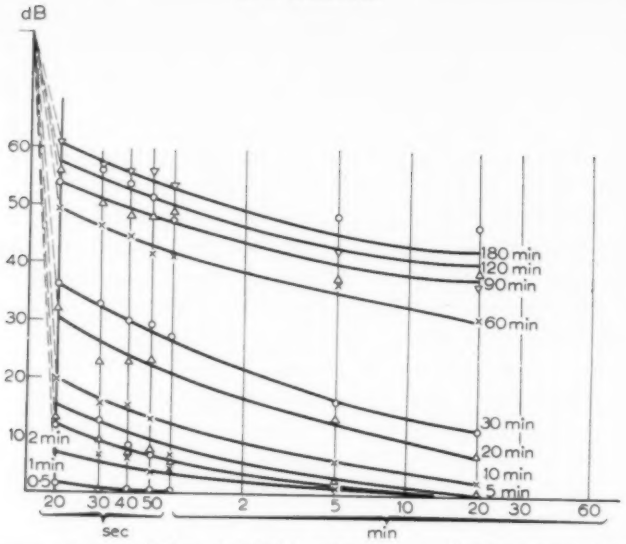

FIG. 7. Curves for audibility threshold restoration. Abscissa—time elapsed after switching off noise (on a logarithmic scale); ordinate—displacement of audibility threshold. Curve parameters are noise durations. Sound signal 2000 c/s.

Figures are given in Table 2 showing the number of decibels to which the audibility threshold had dropped during 1 min at various moments after switching off the noise in different groups of tests. Let us call these figures the values for restoration of the threshold. The value for restoration over the first minute was made apparent by comparison of the thresholds measured during the noise immediately before it was switched off and 60 sec after that moment. The values for restoration in the fifth and twentieth minutes were determined by calculating the changes in threshold values taking place in 1 min at the indicated time, since measurements were not carried out every minute but with intervals for rest. Here we ignored the circumstance which we know from published material [3], and which is evident from Table 2, that the rate of restoration of the hearing decreases proportionately with the time which has elapsed after removing stimulation. This was permissible since we were not interested in the absolute figures for restoration at various moments but in the relationship between the duration of stimulation and the value for the restoration during identical times following the cessation of stimulation.

We can see from Table 2 that the restoration of the threshold in the first minute after the cessation of noise also depends on the duration of stimulation, as was noted earlier on the basis of measurements made 20 sec after switching off the noise (which we called the values for adaptation to silence). At the same time, the value for restoration of the threshold in the fifth, and also in the twentieth, minute fluctuated in the different groups of tests independently of the duration of stimulation.

Thus, though the adaptive capacity in the very first moments after the cessation of noise depends to a great degree on its duration, the gradual restoration of the remaining impairment of the hearing which is observed later on is not so dependent.

This shows the different natures of the processes of alteration in the sensitivity of the hearing which occur at the very first and at subsequent moments after the

TABLE 2. CHANGE OF THRESHOLD WITH TIME AFTER CESSATION OF STIMULATION

Duration of noise, min.	No. of dB to which threshold fell in 1 min, at various moments after noise stimulation		
	In 1st min	In 5th min	In 20th min
0·5	97	Threshold restored earlier	
1	96	Threshold restored earlier	
2	86	Threshold restored earlier	
3	91	Threshold restored earlier	
5	92	Threshold restored earlier	
10	92	2·6	0·23
20	72	3·8	0·53
30	67	3·5	0·33
60	57	2·8	0·46
90	53	3·8	0·06
120	48	4	0·26
180	43	3·7	0·4

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cessation of noise. We can see that in the case where the fatiguing effect of noise has had no effect (after 0·5 min operation) the hearing is fully adapted to silence after 20 sec. It is possible that nerve regulation of the adaptive process proceeds very fast, and in cases where full restoration of aural sensitivity cannot be accomplished in this short time as a result of the fatiguing effect of the noise, this process is accomplished later on by other mechanisms which counteract the remaining impairment of the hearing at an identical tempo independent of the duration of the preceding noise which caused it. Support for this thesis comes from the fact that the effect of using phenamine, as described in the article to which I referred above [15], only affected the audibility thresholds in the moments immediately after the noise. The number of decibels to which the audibility threshold dropped per unit of time during the further restoration did not depend on whether phenamine had been administered.

After more prolonged noise the final restoration of sensitivity came later than after short-term noise; but it would be inaccurate to assert that this is a consequence of a decreased rate of restoration of sensitivity influenced by prolonged noise, for it relates

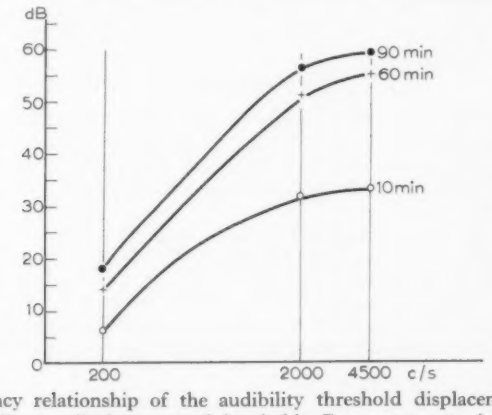

FIG. 8. Frequency relationship of the audibility threshold displacement. Abscissa—frequency; ordinate—displacement of threshold. Curve parameter is noise duration.

only to the very first moments after the cessation of noise. It is most likely that the different times taken by the full restoration of sensitivity in the various tests is due to different values for the adaptation in this initial period.

All the experimental data given in the article were obtained using the same tone of 2000 c/s. What has been stated is, however, applicable for the other tones (200 and 4500 c/s) whose perception in the same conditions was investigated in this work.

As in the work of other authors [3, 17] using pure tones and noise, the present work also involved observation of the frequency relationship of displacement in the audibility threshold when applying sound signals of varying pitches after stimulation by the noise which I used. Fig. 8 illustrates this relationship. It is clear from the graph that maximum displacement of the threshold was observed, other things being equal, where the tone used was at 4500 c/s, and the minimum was where the tone was at 200 c/s.

CONCLUSIONS

This work has thus revealed the following rules for the effects of intense sound stimuli of different durations on the human hearing:

(1) The adaptation of the hearing to silence after the cessation of noise is accomplished at the rate characteristic of the auditory analyzer only in those cases where the duration of stimulation does not exceed a few minutes.

(2) Increase in the duration of the application of noise reduces the rate at which the sensitivity of the hearing is restored in the moments immediately after stimulation.

(3) The change of adaptation to silence in the early period indicated, with increase in the duration of stimulation, follows an exponential law.

(4) The indicated reduction in the rate of adaptation is the essence of the process of increase in the displacement of the audibility threshold after the effect of noise with increase in its duration: this process is evidently also at the basis of the later restoration of the sensitivity of the hearing after more prolonged stimulation.

(5) The differences in the rates of restoration of sensitivity level out 2–5 min after the cessation of noise, after both short-term and prolonged stimulation.

(6) The highest changes in audibility threshold values after the application of noise of the given spectrum occur when a high-frequency tone (4500 c/s) is used; the lowest changes occur with a low-frequency tone (200 c/s).

Translated by J. H. DIXON

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ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF BIOLOGICAL OBJECTS*

EFFECT OF DENATURATION ON THE E.P.R. SPECTRA OF IRRADIATED PROTEINS

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INTRODUCTION

We have previously [1, 2] shown that native protein preparations, frozen while enzymatic processes are occurring, have unpaired electron concentrations of 10^{-7}–10^{-8} M per g dry weight. The widths and shapes of such electron paramagnetic resonance (e.p.r.) lines indicate that electrons are largely delocalized in protein molecules. The unpaired electrons arise because the electronic levels of the substrate, which forms a temporary complex with the enzyme, e.g. in redox reactions, are either (1) directly below the excited electronic levels (conduction band) in the protein, or (2) directly above the electronic ground-state of the protein. Hence, complexes such as substrate–enzyme, prosthetic group–apoenzyme, or metal ion–protein can be considered as impurity semiconductors with either electron or hole conduction, depending on whether we have cases (1) or (2) above; the conductivity can also possibly be of mixed (electron–hole) type. Electron or hole migration was assumed to occur via the peptide hydrogen-bond chains, which are transverse to the main protein chains. Simple quantum mechanical calculations show that even if the potential barriers along such subchains are 2–3 eV high (and deep electron traps are absent; i.e. the impurity levels are suitably located) "tunnelling" can provide the transition frequencies required for the migration.

As would be expected, similar narrow e.p.r. lines result when ionizing radiations act on proteins. It was supposed [2] that the much lower e.p.r. line intensities found in the proteins (as compared with irradiated amino acids and peptides) are the result

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of the much greater probability that the ejected electrons will enter the conduction band and combine with a hole in the first case. Relatively few fall into deep traps at lattice sites from which the conduction band is not accessible. These trapped electrons give very wide e.p.r. lines, which are not detected [3]. The corresponding holes are delocalized on the protein structure and give narrow e.p.r. lines.

If the above ideas are correct then thermal denaturation, which is known to disrupt the hydrogen band network, must eliminate the conduction band and so intensify the e.p.r. lines from irradiated proteins, hyperfine structures also appearing. The present paper is concerned with testing this idea.

METHODS

The apparatus and techniques have already been described [2]. Doses of $1{\cdot}15\times10^7$ r (of cobalt radiation) were used. None of the preparations gave an e.p.r. signal before irradiation. For comparison certain amino acids were irradiated at the same dose. The proteins were denatured in aqueous solution and then freeze-dried. The free paramagnetic particle concentrations were determined by comparison of the signal area with that of a standard diphenylpicrylhydrazyl preparation, diluted with chalk powder. The specimen was placed at the bottom of a cylindrical resonator (H_{111} mode) and the standard on the wall at $\lambda/4$ above the bottom, diametrically opposite the input hole. Corrections were applied for the differing strengths of the high-frequency magnetic field at the two positions. The reproducibility of the relative radical contents was satisfactory.

The oxyhaemoglobin preparation was made as follows. Blood from a donor was centrifuged, the erythrocytes washed three times with 1·2% NaCl solution and then diluted with an equal volume of water and 0·4 times the volume of toluene. After stirring, the mixture was allowed to stand overnight in the refrigerator. The pure oxyhaemoglobin solution (13% concentration) was separated by centrifugation in the morning. The solution was freeze-dried for irradiation; the dry powder dissolved without residue in water and gave the spectrum typical of oxyhaemoglobin. The ichthyocol was presented to us by V. O. Shpikiter (Institute of Biochemistry, Academy of Medical Sciences of the U.S.S.R.), while the pepsin and casein preparations were obtained from Professor Shlygin's laboratory (Institute of Nutrition, Academy of Medical Sciences of the U.S.S.R.). We are indebted to these workers for these preparations.

RESULTS

Amino acids

Glycocol (Fig. 1). A sharp triplet showing very weak satellite peaks symmetrically placed on the wings; signal-centre g-factor $\sim$2·004. Hyperfine splitting: $\Delta H_{1-5} =$

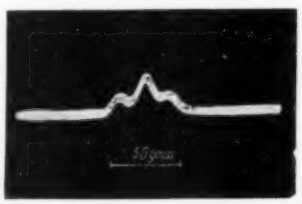

FIG. 1. E.p.r. spectrum of irradiated glycocol.

75 gauss, $\Delta H_{2-4} = 35$ gauss. The main triplet is clearly the result of the unpaired electron interacting with two equivalent protons in a $(CH_2)^+$ radical (intensity ratios 1: 2: 1). The integral intensity corresponds to $\sim 1{\cdot}1 \times 10^{19}$ particles per g of specimen.

Serine (Fig. 2). A complex signal, being a doublet with slight triplet splitting on each peak; *g*-factor 2·007. $\Delta H_{1-6} = 66$ gauss; $\Delta H_{2-5} = 37$ gauss; $\Delta H_{3-4} = 18$ gauss. The integral intensity corresponds to $\sim 1{\cdot}4 \times 10^{19}$ particles per g.

Histidine (Fig. 3). A triplet with broad components and an anomalous intensity relation (1: 3: 1); *g*-factor 2·004, $\Delta H_{1-3} = 72$ gauss. About 7×10^{18} free radicals per g.

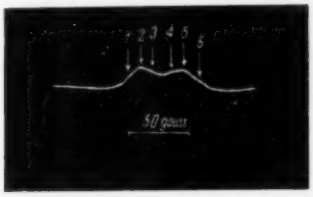

FIG. 2. E.p.r. spectrum of irradiated serine.

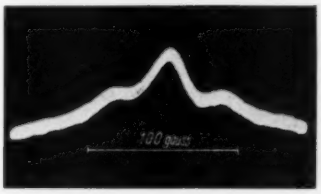

FIG. 3. E.p.r. spectrum of irradiated histidine.

Thus irradiated amino acids give broad intense e.p.r. lines with hyperfine structures, as before [2]. At $1{\cdot}15 \times 10^7$ r ($1{\cdot}9 \times 10^{19}$ ionizations per g) about 10^{19} free radicals are formed per g.

Native protein preparations

The proteins used were oxyhaemoglobin, casein, ichthyocol and pepsin. In all cases the native preparations gave narrow e.p.r. lines after irradiation, as before [2]: widths 1·5–4 gauss (casein 4–6 gauss). The intensities corresponded to $1\text{–}2 \times 10^{16}$ paramagnetic particles per g, or less (oxyhaemoglobin, pepsin).

Denatured proteins

Oxyhaemoglobin. The protein was denatured by heating the aqueous solution in a water-bath (a) at 73°C for 2 min (56 per cent coagulation) (b) at 100°C for 15 min (100 per cent coagulation) (c) at 100°C for 30 min. The specimens were frozen and dried *in vacuo* immediately after denaturation. In all cases doublet e.p.r. signals were seen after irradiation, the intensities increasing with the extent of denaturation. Signal-centre *g*-factor 2·008; $\Delta H_{1-2} = 16$ gauss. The concentrations (per g) corresponding to the integral intensities were (a) $\sim 6 \times 10^{16}$ (b) $\sim 1 \times 10^{18}$ (c) $\sim 3 \times 10^{18}$. Fig. 4 shows the signal from preparation (c).

FIG. 4. E.p.r. spectrum of irradiated denatured oxyhaemoglobin.

Pepsin. The protein was denatured by boiling in distilled water for 1 hr. The irradiated material gave a weak signal with a doublet structure of shape and width analogous to that above.

Ichthyocol. The protein was denatured by boiling for 1 hr. The irradiated preparation gave a sharp doublet (Fig. 5), not distinguishable from that given by denatured oxyhaemoglobin. The free radical concentration was $\sim 7 \times 10^{18}$ per g.

Casein. As for ichthyocol (above), g-factor 2·005 and $\Delta H_{1-2} = 16$ gauss. Radical concentration $2{\cdot}5 \times 10^{18}$ per g.

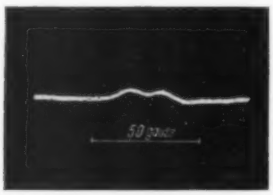

FIG. 5. E.p.r. spectrum of irradiated denatured ichthyocol.
FIG. 6. E.p.r. spectrum of irradiated denatured casein.

DISCUSSION

In all cases thermal denaturation produced a comparatively broad post-irradiation doublet e.p.r. signal with about a 16 gauss hyperfine splitting instead of the single narrow signals from the native proteins. The free radical concentrations produced by irradiation were increased by denaturation by factors of 100–500. The effect of degree of denaturation on resultant radical concentration was demonstrated with oxyhaemoglobin.

FIG. 7. E.p.r. spectrum of irradiated glycylglycine.

The signal shape and hyperfine splitting found in irradiated denatured proteins are completely analogous with those found in irradiated crystalline glycylglycine (Fig. 7). In this latter case the doublet is caused by the unpaired electron (localized on the O atom) interacting with the NH-group proton [3], which proton forms an intermolecular hydrogen bond. We may suppose the e.p.r. signals from the denatured proteins to have a similar origin.

Thermal denaturation replaces the long hydrogen-bond subchains by chaotic hydrogen bonds which do not form a single system. The conduction channels via which the ejected electrons combine with the holes are thereby destroyed, and the free radical concentrations hence raised. We consider that our data indicate that electron conduction dependent on the hydrogen-bond network is present in native proteins.

SUMMARY

(1) The effect of prior thermal denaturation on the e.p.r. spectra of irradiated dry preparations of oxyhaemoglobin, pepsin, ichthyocol and casein has been studied.

(2) As a result of such denaturation, post-irradiation doublet splitting (the same as found with crystalline glycylglycine) is observed in the e.p.r. spectra.

(3) Denaturation increases the radiation-induced free radical concentrations by several hundred times.

(4) The observed effects are given a structural interpretation.

Translated by J. E. S. BRADLEY

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ULTRA-VIOLET MICROSCOPY AND CYTOPHOTOMETRY OF NORMAL AND X-IRRADIATED BONE-MARROWS*

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MUCH of the study of the effects of ionizing radiations on nuclear metabolism in various tissues indicates that changes in the nucleic acids are the root cause of radiation injury. Many workers have observed radiation-induced structural changes in the nucleic acids, and some changes of content. Radiation has been shown to depolymerize nucleic acid solutions [1–4]. The same has been found with nucleoproteins from irradiated animals [5, 6] and plants [7]. Changes in nucleic acid content and reductions in turnover rate have been observed in irradiated tissues [8–14]. But studies on homogenates of cells of very different origins and functions, or on degree of damage, cannot show whether the changes in nucleic acid content precede or merely accompany cell destruction (i.e. whether the primary damage is to the nucleic acids). The changes in the nucleotides present in many materials, including nucleic acid precursors or metabolites, are also important. Cytochemical studies [5, 15, 16] have revealed changes in RNA content in the cytoplasm of cells from irradiated animals. The data on nucleic acids in nuclei are contradictory and inaccurate. The nucleus is also very sensitive to ionizing radiations. There are no data on changes in free nucleotides in cells.

The present paper deals with determining the changes in nucleic acid and nucleotide contents of nuclei from various bone-marrow cells in mice after a minimum

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absolute lethal X-ray dose (700 r). Only cells not externally damaged were studied, of morphology not different from normal. This was done to try to show whether the changes in nucleic acid and nucleotides in the nuclei precede the morphological degeneration. Most of the work was done by a quantitative cytochemical method. Many marrow cells were first studied visually with an ultra-violet microscope.

ULTRA-VIOLET MICROSCOPY OF NORMAL AND IRRADIATED BONE-MARROW

Visual studies were made on marrow smears fixed with formaldehyde vapour. Characteristics such as cell and nuclear shape and dimensions, and intensity of cytoplasms and nuclear staining using basic and acid stains (basophilia and oxyphilia), were studied on normal stained preparations. Unstained cells or tissue sections were studied in the ultra-violet microscope, the images being produced by ultra-violet absorption by the cell materials. In differentiating cellular forms in the ultra-violet we mainly used the first two characteristics. The basophilia index can also be used (although to a lesser extent) because this property is dependent on nucleic acid being present.

Only the following cellular forms, clearly distinguishable by nuclear size and shape (and sometimes by cell shape), were used: reticulocytes, haemocytoblasts, myeloblasts, myelocytes, special leucocytes (neutrophils), erythroblasts and normoblasts. Twenty fields of view were examined on preparations for each period after irradiation (giving 80–400 cells of each generation at each period). The cell numbers were recorded and the strength of the ultra-violet absorption noted.

Normals. The nuclei of most haemocytoblasts and myeloblasts absorb ultra-violet very strongly, and so appear dark. The absorption varies between cells, about half having nuclei showing very strong absorption and therefore appearing black. Many absorb less strongly but also appear dark. Some 5–7 per cent have light nuclei (absorption weak).

The myelocytes are about the same; but the ratio of light to dark nuclei is somewhat different in neutrophils. Few nuclei in the latter appear black, most being dark (absorption average) but with some light ones. The numbers are about the same as in less differentiated forms. Erythroblasts show the same dark: light ratio. In normoblasts the nuclei are very small, the nuclear content being concentrated in a small volume, and the absorption mostly being very strong (dark and black nuclei); but some 5 per cent are light.

Hence the nuclei of most cells (90–95 per cent) absorb ultra-violet strongly, the rest (some 5 per cent) absorbing much more weakly (Fig. 1(a)).

Five minutes after irradiation (700 *r*). The dark: light ratio was already altered. By this time, 16 per cent of the haemocytoblasts and 25 per cent of the myeloblasts had light nuclei, the proportion of black having fallen from 40–45 per cent in the normal to 15 per cent in the irradiated cells. The fraction of light nuclei in neutrophils and myelocytes had risen to 15–20 per cent of the total (570). The ratio was unchanged in the erythroblasts and normoblasts, but these forms are less suitable for visual study as the nuclear volumes are small and the absorber concentrations high.

Six hours after irradiation. Light nuclei predominate in all forms, save normoblasts (Fig. 1(*b*)). The fractions are: haemocytoblasts, 70 per cent; myeloblasts,

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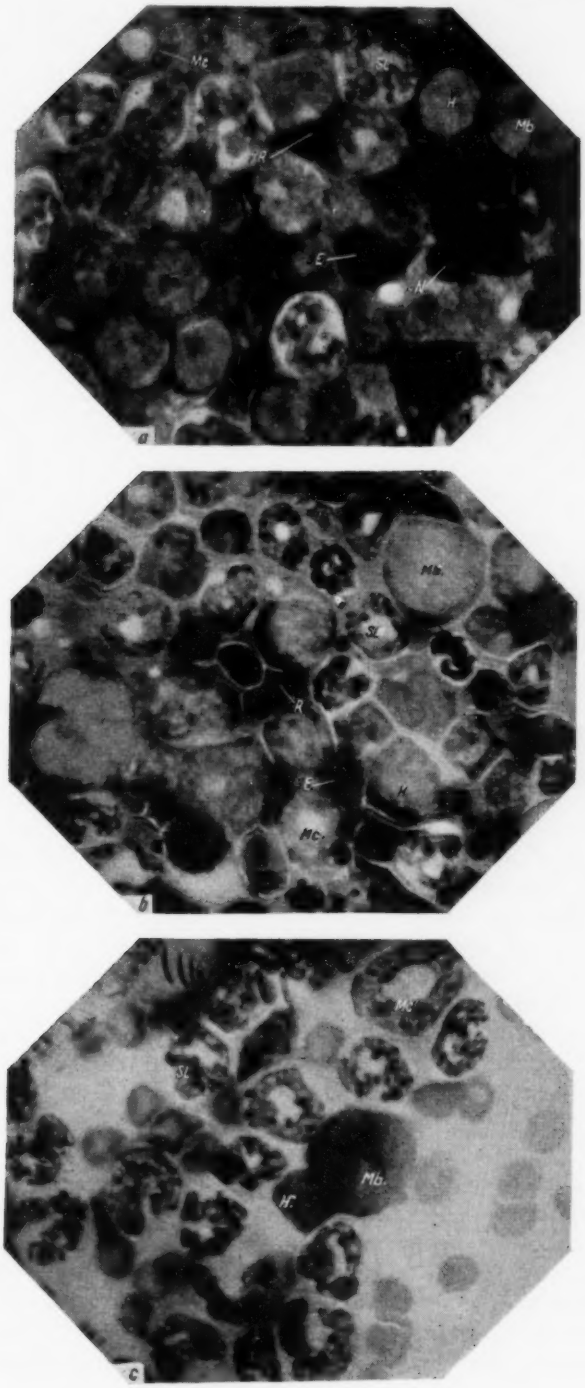

FIG. 1. Ultra-violet photomicrographs of bone-marrow. Absorption at $\lambda = 265$ mμ by nucleic acids and nucleotides: (*a*) normal, (*b*) at 6 hr after irradiation, (*c*) at 24 hr after. *R*—reticulocyte; *H*—haemocytoblast; *MB*—myeloblast, *MC*—myelocyte; *SL*—special leucocyte (neutrophil). *E*—erythroblast, *N*—normoblast. Objective, quartz ×58; N.A. 0·8; eyepiece, K × 8. Magnification ×1800.

about 80 per cent; myelocytes, 97 per cent; erythroblasts, about 70 per cent; all specialized leucolytes having light nuclei. The only form showing dark predominant over light was the normoblast, but this form is difficult to study. The visual observations thus show reduced ultra-violet absorption within a few minutes of irradiation, the effect growing stronger with the passage of time.

Two causes may reduce the absorption, namely increase in cell and nuclear volumes, or reduction in absorber content (wavelength used 265 mμ). The cells do indeed change in size, but only slightly even after more than 6 hr (Barakina [17]; also our data). We also separated the giant cells into special groups. Thus the drop in absorption is mainly due to loss of absorbing material.

The second section of the work was concerned with elucidating some of these changes, quantitative cytochemistry being used.

QUANTITATIVE CYTOCHEMICAL MEASUREMENTS (CYTOPHOTOMETRY)

The optical density D, which is proportional to concentration, is given by

$$D = \log (I_0/I)$$

where I is the light intensity transmitted by the structure, and I_0 the same when the absorber has been removed (background). The optical density has to be measured to calculate the concentration c (g/cm^3)

$$c = D/xd$$

d being the layer thickness (cm) and x the absorption coefficient (a constant for the substance). The quantity Q is given by $Q = cV = (D/xd)\,Sh$. For smears $d = h$ and $Q = DS/x$ (g), where V is the cell volume, S the cell area in the smear and h the cell height (layer thickness).

A photographic method previously described by one of us [18, 19] was used to determine $\log I_0$ and $\log I$. Ultra-violet photomicrographs were taken, monochromatic light of wavelength 265 mμ being used (the monochromator being a grid type with 1200 lines/mm). The optics were a quartz immersion objective, ×58, N.A. 0·8 and a ×8 eyepiece. The plates used were "Spectral, type I".

When dry smears are fixed in formaldehyde vapour the substances showing ultra-violet absorption at 265 mμ are retained, namely RNA, DNA, nucleotides (monomers and higher forms), aromatic amino acids (free and combined in proteins) and some vitamins. The distribution and total amount of all the above materials are represented on the photograph. When several areas in the smear had been photographed the quartz coverslip was removed and the smear treated with cold (0–1°C) trichloroacetic acid for 5 min. Free aromatic amino acids, nucleotides, vitamins and certain other substances are thereby removed. The latter substances are of no interest to us because they do not absorb at 265 mμ. The extraction is complete in 4–6 min. In all cases, 5 min was used. After careful washing, the smear was replaced in the microscope under the coverslip and again photographed. The absorption in the second case is due to the nucleic acids and aromatic amino acids in the proteins. Finally, a third set of photographs was taken after treatment with hot trichloroacetic acid (90°C) for 5 min, this set showing the absorption from some proteins. The three sets thus enable one to determine the optical densities due to acid-soluble substances and nucleic acids, as shown below (I denotes the light transmitted by the preparation):

Before treatment	Nucleotides DNA and RNA Aromatic amino acids (free) Aromatic amino acids (in proteins) Vitamins	I'
After treatment with cold trichloroacetic acid	RNA and DNA Aromatic amino acids in proteins	I'_0 and also I''
After treatment with hot trichloroacetic acid	Aromatic amino acids (in proteins)	I''_0

$D'_0 = \log (I'_0/I')$ gives the optical density due to acid-soluble substances. These are mostly nucleotides,* since the free aromatic amino acids and the vitamins have much less absorption. We may thus for brevity call the acid-soluble fraction the nucleotide one, provided we remember that it contains traces of other substances. $D''_0 = \log (I''_0/I')$ gives the nucleic acid contribution.

More than 2000 bone-marrow cells were studied, from normal white mice (16 animals) and from animals taken at 5 min and 1, 6, 24 and 72 hr after irradiation (8 used in each case). The minimum absolute lethal dose (700 r) was used, the irradiation conditions being 150 kV, current 3 × 10 mA, filters 0·5 mm Cu + 0·75 mm Al, irradiation time 14 min 36 sec, dose-rate 48 r/min.

QUANTITATIVE CYTOCHEMICAL ANALYSIS FOR NUCLEIC AICDS AND NUCLEOTIDES IN BONE-MARROW CELLS AFTER IRRADIATION

Figs. 2–4 show the results for the nucleic acid and acid-soluble (nucleotide) fractions for normal cells. Each point is the mean of those for 20–40 cells. Figs. 2–3

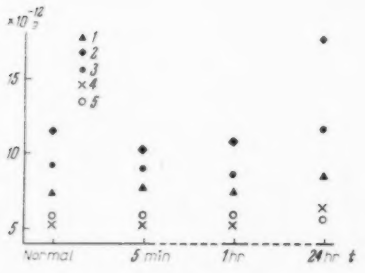

FIG. 2

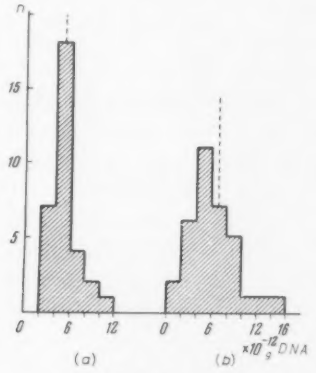

FIG. 3

FIG. 2. Nucleic acid contents in the normal bone-marrow and after X-irradiation. (1) Haemocytoblasts, (2) myeloblasts, (3) myelocytes, (4) neutrophils, (5) erythroblasts. t = time after irradiation.

FIG. 3. Amounts of DNA in neutrophil nuclei. (*a*) Normal, (*b*) 24 hr after irradiation; n = number of cells. The dashed lines indicate the mean values.

* Roskin and Brodskii [20] have earlier determined nucleotides in cytological objects. Lagerstted [21] has determined nucleotides in acid extracts of tissue slices (freeze-dried, cold perchloric acid) by chromatography. The nucleotides from isolated animal cell nuclei have recently been determined by chromatography [22].

show that the amounts of the nucleic acids in apparently undamaged cells are unchanged after the first few hours. The amounts rise somewhat after 24 hr in the less differentiated myeloid cells—haemocytoblasts, myeloblasts and myelocytes.

The fraction soluble in cold trichloroacetic acid shows considerable changes after the irradiation. Its optical density is mostly due to nucleotides (metabolites from nucleic acid, ATP, etc.) and the amounts are quite large in normals. The nucleic acid: nucleotide ratios in the nuclear zone of myeloid series cells are quite large: haemocytoblasts, 1·5; myeloblasts, 1·5; myelocytes 1·8; neutrophils 1·6. The nucleotide contents of erythroblasts and normoblasts are relatively lower, the ratios being 2·5–3·0. The nucleotide contents of nuclei in normal cells correlate closely with the nucleic acid contents and nuclear size. Thus, we may suppose that much of the nucleotide results from nucleic acid metabolism.

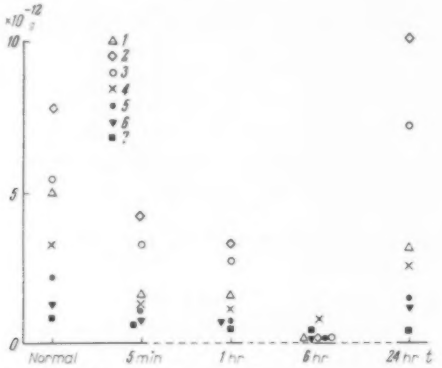

Fig. 4. Amounts of nucleotide in bone-marrow cells, normal and after irradiation. (1)–(5) as for Fig. 2, (6) normoblasts, (7) reticulocytes; t = time after irradiation.

Immediately after irradiation the nucleotides drop sharply in amount, as Fig. 4 shows, each point being the mean for 30–70 cells. The differences, of which we shall have more to say later, are statistically reliable. The amounts fall by a factor of about 2 in all cells except the reticulocytes by 5 min after irradiation, and may do so earlier. The levels are as low, or somewhat lower, at 1 hr. The reduced level persists only in the reticulocytes and neutrophils at 6 hr, the level being zero in all other cell types. At 24 hr the normal level is regained. Giant cells appear in the bone-marrow at 24 hr, these being myeloblasts and myelocytes with increased nucleotide and nucleic acid contents, but their numbers are small. These cells are discarded in calculating the means for 24 hr as the increases in the contents are due to the cell sizes.

DISCUSSION

We must first decide what nucleic acids were removed in the hot acid fraction. Purines and pyrimidines, which are present in RNA and DNA, absorb at 265 mμ. The hot trichloroacetic acid removes both, so we have to determine the DNA and RNA contributions to the nuclear absorption. In allowing for the RNA we must remember that in smears we have also cytoplasmic RNA above and below the nucleus. This is particularly important in forms with basophilic cytoplasm (haemocytoblasts

and myeloblasts). But certain observations indicate that this does not occur. For instance, Davis [23] has shown that the layers of cytoplasm above and below the nucleus are very thin in connective tissue. The same applies to our measurements on bone-marrow; these very thin layers have scarcely any effect on the optical density. Our data also indicate that the nucleic acid content in the nuclear zone is much higher, not merely in the highly basophilic haemocytoblasts and myeloblasts, but also in the much less basophilic myelocytes. In myelocytes the cytoplasm contains less RNA than in haemocytoblasts, but the nucleic acid in the nuclear zone is much higher. Hence RNA interference is absent. Finally, the interesting work of Kirpichnikova *et al.* [15] shows that the intensity of pyronin staining in the bone-marrow cytoplasm falls in the first hours after irradiation. The same has been observed in plant cells [7]. The reduced staining may be assigned to reduced cytoplasm RNA. Our measurements show that the nuclear nucleic acid is actually raised; this also indicates that RNA interference is absent.

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Treatment of the smears with a solution of crystalline ribonuclease (0·1% solution, 37°C, 1 hr) reduced the nuclear optical densities in haemocytoblasts and myeloblasts, the nucleic acid contents falling from 7·5 to $5{\cdot}9\times10^{-12}$ g and 11·3 to $6{\cdot}2\times10^{-12}$ g respectively, the latter figures being comparable with the nuclear nucleic acid of erythroblasts ($5{\cdot}6\times10^{-12}$ g) and neutrophils ($5{\cdot}4\times10^{-12}$ g). Many workers have shown that the mean DNA in diploid mouse cell nuclei varies from 5 to 7×10^{-12} g (for a review see [13]).

The above figures therefore lead to the following conclusions:

(1) DNA is the main nuclear nucleic acid in erythroblasts and neutrophils.

(2) The DNA contents of the above forms are unaltered by irradiation, so it is improbable that the constant nucleic acid contents of the other bone-marrow species is due to a rise in RNA plus a comparable fall in DNA.

(3) The DNA contents of different generations of marrow cells are about the same, the differences in nucleic acid content (Fig. 2) being due to variations in the RNA.

The myeloblasts and myelocytes show increased nuclear nucleic acids at 24 hr after irradiation. This might be due to more numerous mitoses at 20–24 hr after irradiation [17, 24]. The DNA content is doubled at interphase [25, 26]. But although the mitoses are much more numerous at 24 hr than at 3–10 hr they are still below normal, while the nucleic acid content at 24 hr is above normal.

The following two assumptions are therefore proposed:

(1) Not all the old cells are dividing at 24 hr but only some; the number of mitoses in the latter may be above normal. Barrow's [24] and Barakina's [17] data support this; the erythroid series was found to be much more sensitive than the myeloid. We only found increased nucleic acid in the myeloblasts and myelocytes.

(2) The rise in nucleic acid is due to causes related to cell pathology, and not merely to extra mitoses. This rise at 24 hr may be related to increased mitotic activity; it may be explained by new reticulocytes, which are little damaged, appearing (Fig. 4) as these remain in large numbers; or there may be a similar effect with some other type of cell. There are no (or very few) new cells in the first few hours after

irradiation, the mitotic index being nearly zero [24]. The first mitoses appear at 10 hr, the maximum being at 20 hr, the level remaining high until 30 hr, falling thereafter to zero at 65 hr. The mitotic activity and nucleoprotein curves coincide.

The data indicate that the radiation depolymerization of the nucleic acids, observed by many workers, is not very great, the substances being still relatively highly polymerized. The amounts of poly- or mono-nucleotides extracted from undamaged cells by cold acid actually fall rather than rise after irradiation. Further, the depolymerization to mononucleotides only occurs in damaged cells, which we did not study in the early post-irradiation period.

The changes in nuclear fluorescence after irradiation are usually said to be due to nucleic acid depolymerization [27, 28]. The changes may possibly be really due to fall in the amount of nucleotides.

The biochemical evidence for a fall in bone-marrow DNA is probably to be explained as a reduction in the number of cells. The nucleic acid is not reduced in amount in undamaged cells and damage to the normal structure is not related to changes in the nucleic acid content occurring long before the damage. The contents remain high up to 24 hr in bone-marrow cells showing no visible damage, although the numbers of such cells fall rapidly to a very low level at 24 hr. At 72 hr, when most of the cells show marked signs of degeneration, the amounts of nucleic acid drop sharply from $5–12\times10^{-12}$ g (normal) to $1{\cdot}5–2\times10^{-12}$ g in the damaged cells. Of course, we cannot categorically assert that the DNA is completely unaffected by the radiation in the apparently undamaged cells; qualitative changes (e.g. in the molecular structure) possibly occur in the DNA, but they could not be observed by our methods.

The marked reduction in the nucleotides (which are nucleic acid metabolites and precursors) must imply a drop in the nucleic acids also. But since we only studied undamaged cells (at 24 hr), while the fall occurs only in damaged ones, we could not establish such a relation.

SUMMARY

(1) The mean amounts of DNA in the nuclei of various cells from normal bone marrow are about equal at $5{\cdot}5–6{\cdot}2\times10^{-12}$ g. The amounts of RNA in the less differentiated cells are higher than in adult ones.

(2) The mean amounts of material (mostly nucleotides) soluble in cold trichloroacetic acid and found in the nuclei of various bone-marrow cells are fairly large. The nucleic acid: nucleotide ratio is fairly constant within the myeloid series (1·5–1·8) and within the erythroid (2·5–3·0).

(3) Minimum absolute lethal X-ray doses to mice (700 r) produce no reduction in the nuclear nucleic acid in undamaged marrow cells (haemocytoblasts, myeloblasts, myelocytes, neutrophils, erythroblasts) at 24 hr after.

(4) The amounts of nucleotide in these undamaged cells fall to half immediately after the irradiation, reaching zero at 6 hr in haemocytoblasts, myeloblasts, myelocytes, erythroblasts and normoblasts. The amounts are raised at 24 hr.

(5) The reticulocytes show little fall in nucleotide, the level remaining much the same throughout.

(6) The nucleic acid contents of damaged cells fall by factors of 3–5, and the nucleotides to zero, at 72 hr, when the cell structures break up.

(7) Our observations show that radiation damage to cells is probably related to disturbance in nucleic acid metabolism (one of which disturbances is the fall in nucleotide content) and not to reduction in the amounts of nucleic acid.

This work was performed in the Radiobiology and Cytology Laboratories at the Institute of Animal Morphology, Academy of Sciences of the U.S.S.R. We offer our thanks to the directors of these laboratories, Professors E. Ya. Graevskii and G. K. Krushchev, for their assistance.

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CELL PROTECTION BY FATTY MATERIALS AGAINST RADIATION DAMAGE*

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FATS and lipids have many functions in organisms. They are present in cell structures such as the mitochondria and microsomes, the plasmalemma and tonoplast. In combination with proteins (lipoproteins) they are present in the cytoplasm. Microscopic and submicroscopic lipoprotein and liponucleoprotein structures play a large part in enzymatic and sorption processes and are intimately connected with active transport of materials across cell membranes and with intracellular distribution processes. Fats and lipids frequently also accumulate in the free state in the protoplasm and then act as reserves; the amounts can rise if metabolism is disturbed.

Lipid accumulation may have a double significance. It primarily denotes disturbances in oxidative processes or carbohydrate metabolism, and is sometimes related to deficiencies in nitrogen intake. If much fat and lipid is deposited in the cell it cannot but affect the redox situation, since fats and lipids are oxidizable. Some [1, 2] consider fat accumulation related to reduction of the metabolic rate and to the cell adopting a resting state.

Nadson [3, 4] was one of the first to observe that X-irradiated yeast cells become rich in fat. This effect was subsequently observed in both lower and higher organisms [5–7]. Very marked fat, lipid, and steroid accumulation has been found in microorganisms which continue their metabolic activity after irradiation [8, 9]. This fat accumulation may possibly be a protective reaction following irradiation, since the ease with which lipids are oxidized, and the large amounts accumulating in the irradiated organism, lead us to suppose that they may regulate the redox situation in the cell and hence increase its resistance to radiation damage. It has been claimed that animals are less radio-sensitive if first fed with fat-rich feed; cotton-seed oil (50 per cent linoleate) added to the food, increased the radio-resistance. This seemed to indicate that the protective action of cotton-seed oil was due to the essential fatty acids it contains [10–12]. It has also been claimed that those animals which survive longer after irradiation have higher lipid levels than those which die rapidly [13]. It is also very likely that the steroids have a part in stimulating recovery [14].

We studied the protective action of fat on *Endomyces magnusii*, which is convenient for the purpose because the fat contents of the cells can be adjusted by varying the culturing conditions. Rider's medium (modified) was used to produce fat enrichment or depletion. If carbohydrate sources predominate in the medium the organisms are fat-rich, whereas if the nitrogen is high they are fat-poor.

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The composition of the Rider's medium (using glycocol instead of ammonium sulphate) which gives medium fat contents is (per litre of tap-water) as follows:

Glycocol	1·7 g
$MgSO_4.7H_2O$	0·7 g
NaCl	0·5 g
$Ca(NO_3)_2$	0·4 g
KH_2PO_4	1·0 g
K_2HPO_4	0·1 g
Sucrose	20 g
Agar-agar	20 g
40% yeast autolyzate	2·5–5 cm^3
pH	5·4–5·6

The amounts of carbohydrate, nitrogen and phosphate were varied to alter the fat contents, all other components remaining as below (Table 1), four types being used.

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TABLE 1. TYPES OF MEDIUM USED

Type	Medium	Amounts in g/l			
		Glucose	Sucrose	KH_2PO_4	K_2HPO_4
I	1	8·5	4	1	0·1
	2	0·3	40	0·1	0·01
II	1	8·5	4	0·1	0·01
	2	0·3	40	0·1	0·01
III*	1	3	1	0·5	0·05
	2	0·15	20	0·5	0·05
IV	1	8·5	4	1	0·1
	2	0·3	40	1	0·1

* Here distilled water plus Barkholder's microelement mixture was used.

In each case medium 1 was used to produce low fat contents, 2 to produce high ones. In all cases 0·5 per cent of yeast autolyzate was used to provide vitamins; *Endomyces magnusii* does not form cell chains on media containing 0·25–0·5 per cent yeast autolyzate: this is important for colony counting. These media were equal as regards biomass produced, as Table 2 shows.

Equal volumes of aqueous cell suspension were placed in Petri dishes containing media 1 and 2. The dry weight of the cell culture was determined after two days, on 20 cm^3 of medium. Under the microscope cells grown on medium 1 showed a few small fat droplets, while those from 2 showed numerous fat inclusions (Figs. 1 and 2). The cells were filtered off, washed and irradiated as 2% suspensions in water, using a ^{60}Co source, dose-rate 4800 r/min, at doses of 60 and 100 kr. The cell viabilities were determined by counting the colonies growing on a complete nutrient medium

TABLE 2. WEIGHTS GROWN ON MEDIA 1 AND 2

Expt.	Plate No.	Dry weight, mg	
		Medium 1	Medium 2
I	1	32·7	46·2
	2	34·4	46·1
	3	31·3	54·7
II	1	54·1	62·9
	2	56·3	53·7
	3	55·9	47·2

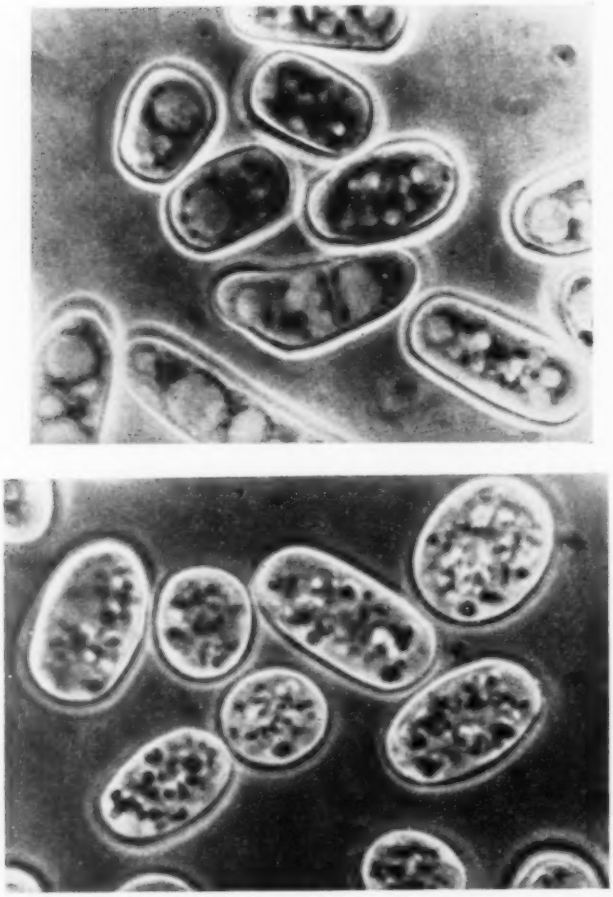

FIG. 1. *Endomyces magnusii* cells with little fat (top) and much fat (bottom). Phase-contrast microscope.

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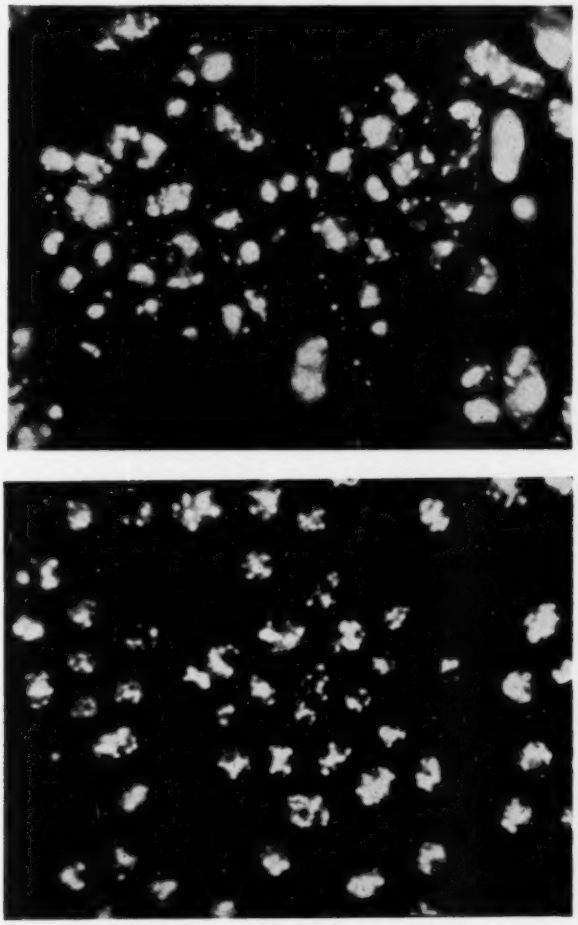

FIG. 2. *Endomyces magnusii* cells with little fat (top) and much fat (bottom). Fluorescence microscope.

(wort agar), the results being given in Table 3 (means of 16 experiments). The fatty cells were on average 30 per cent more viable.

The highest viabilities were found in fatty cells grown on media deficient in nitrogen and rich in carbon, but containing less phosphate. If the C: N ratio was altered while keeping the phosphate normal no definite changes in radio-resistance

TABLE 3. RELATIVE POST-IRRADIATION VIABILITY CHANGES

Type	Fat-poor cells	Fat-rich cells
I	100	138·2
II	100	127·6
III	100	119·7

were found. We thus supposed phosphorus to play some part in protection; special experiments were done, using high and low phosphorus media, to test this hypothesis.

In one series the cells were grown on normal medium (1 g KH_2PO_4, 0·1 g K_2HPO_4) and on medium containing ten times less phosphate (both types). The Rider's medium was made up from distilled water plus essential trace elements. In a second series the cells were depleted of phosphorus by being grown on liquid Rider without phosphate in a shaker for 18 hr, enriched ones being produced by using the same medium but adding 0·5 per cent KH_2PO_4. These cells were irradiated and their viabilities determined. Various species of yeast were used here: *Saccharomyces Ludwigii*, *Torula utilis* and *Endomyces magnusii*. No differences in viability between the phosphorus-rich and the phosphorus-poor cells were detected; phosphorus as such does not affect the radio-sensitivity, but does so in combination with the other major elements. In our experiments, reducing the phosphate while altering the C: N ratio facilitates fat and lipid formation, and thus reduces the radio-sensitivity.

The nature of the protective action of fats has not been elucidated, but there are grounds for supposing it to be related to their oxidizability; the fats react with oxidants and protect vital cell structures. To check this we measured the oxidation rates of fat-rich and fat-poor cells, using the oxygen uptake rate in a Warburg apparatus in the usual way, with and without adding an oxidizable substrate (1% sucrose). Table 4 gives the results. It is clear that the fat-rich cells are some three times less active than the fat-poor when the substrate is provided, so fat-rich cells show an O_2

TABLE 4. OXYGEN CONSUMPTION ($Q(O_2)$) OF *Endomyces magnusii* CELLS WITH LOW AND HIGH FAT CONTENTS

Expt.	With substrate		Without substrate	
	Low fat	High fat	Low fat	High fat
1	78·1	22·2	4·5	18·8
2	77	31·8	6·9	16·3
3	46·9	21·2	2·7	9·1
4	75·2	20·2	9·6	22·8
5	57·6	13	6·31	14·2

NOTE. In Tables 4 and 5, $Q(O_2)$ denotes the volume of oxygen in mm^3 absorbed per hour per mg dry weight.

consumption almost three times as great as that of fat-poor ones. The fat reserves thus influence the oxidation considerably; we then studied how the O_2 absorption varies with fat content, the results being given in Table 5 (no substrate present). It is clear that $Q(O_2)$ is only 25 per cent up after irradiation with the fat-rich cells, the rise being 85 per cent in the fat-poor. The fat reserves influence oxidation in irradiated cells considerably.

In this connexion, oxidation-blocking in irradiated fat-rich cells is of interest. Fat oxidation by atmospheric oxygen or irradiation is known to be much reduced by certain anti-oxidants, such as the tocopherols, vitamin A, ascorbic acid, derivatives of phenol and catechol, certain aromatic amines, etc. The lipid-soluble anti-oxidants such as tocopherol (vitamin E) and ionol are the most effective [15–17].

TABLE 5. $Q(O_2)$ FOR *Endomyces magnusii* CELLS OF LOW AND HIGH FAT CONTENTS AFTER IRRADIATION AT 60 kr (MEANS OF THREE SERIES)

	Fat-poor		Fat-rich	
	Normal	Irradiated	Normal	Irradiated
Q (O_2)	6·2	11·5	15·4	19·3
Per cent of normal	100	185·5	100	125·3

In our experiments on viability changes we used 24 hr *Endomyces magnusii* cultures of high fat content; these were kept in a tocopherol solution (50 mg/l.) in a shaker for 18 hr prior to irradiation. The tocopherol emulsion was produced by dissolving the tocopherol in 96% alcohol and the resulting solution was diluted with water to the required concentration, the alcohol being then distilled off. Identical cells kept in water served as controls. After 18 hr the cells were γ-irradiated as above (60 kr) and cultured to determine viabilities. Table 6 gives the results.

TABLE 6. MEAN PER CENT VIABILITY OF CELLS IRRADIATED IN PRESENCE OF α-TOCOPHEROL (RATIO TO CONTROL)

Expt.	Controls	With tocopherol	
1	100	78·8	
2	100	69·5	Mean 73·7
3	100	78·9	

These experiments showed that the tocopherol-treated cells were on average 26 per cent less viable than the controls. This indicates that the α-tocopherol reduces the fat oxidation on irradiation and so reduces the radio-resistance. We thus suppose that the fats at least partially protect more vital cell components from oxidation, thus increasing the radio-resistance. Hence fat-rich cells are less radio-sensitive. It is also quite possible that the increased fat and lipid formation is a protection response to the production of oxidizing substances on irradiation.

The above discussion relates to fats and lipids not directly involved in the structural organization of the cells, since the structural fraction is very constant in amount, being bound to proteins and nuclear components. Hence, the oxidative processes occurring in the lipids must inevitably cause damage to the cell structures, i.e. increase the radio-sensitivity. Hence, the bound structural lipids must exert an effect opposed to that of the lipid reserves. This topic requires a special investigation.

CONCLUSIONS

The data on *Endomyces magnusii* give rise to the following conclusions:

(1) Altering the ratio of C and N sources in a phosphate-poor medium produces cells which differ widely in reserve lipid and fat content; α-tocopherol affects the viabilities of irradiated cells.

(2) Fat-rich cells oxidize substrates only at about one-third of the rate shown by fat-poor ones, rates of oxidation of internal reserves being the reverse.

(3) On average, fat-rich cells are 30 per cent more radio-stable than fat-poor.

(4) Irradiated cells metabolize their internal reserves less rapidly if rich in fat.

(5) Anti-oxidants reduce the viability of irradiated cells by about 25 per cent on average.

(6) The large fat and lipid reserves have a marked effect on intracellular oxidation rates. This explains why reserve fat renders the cells less radio-sensitive.

In conclusion I express my indebtedness to Professor M. N. Meisel' for guidance in this work.

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LETTERS TO THE EDITOR

THE PROBLEM OF SO-CALLED "SPONTANEOUS" EXCITATIONS OF THE CENTRAL NERVOUS SYSTEM*

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THE central nervous system (c.n.s.) is constantly maintained in an active state in the absence of specific stimuli by a number of constant or long-acting afferent influences on the exteroceptor and interoceptor systems. These constant factors may be stable and prolonged external stimuli or, no doubt, the actual vital processes of the body—cyclical or continuous (e.g. the activity of the respiratory apparatus, variations in the blood pressure, muscle tone, etc.). Central excitation may arise both as the direct result of these factors and as a result of the action of circuits within the c.n.s. [1]; in the latter case the "triggering" of this action can still be causally related to the stimulation of afferent components.

Work carried out in recent years on the role of the reticular formation of the medulla and other parts of the brain [2] has enabled us to obtain a more concrete idea of the significance of afferent impulses in the activation of various parts of the c.n.s. The natural activity of the c.n.s. arising in this way as observed experimentally has sometimes been termed "spontaneous" [3].

The continuous "ordinary" stimulation of the c.n.s. may probably be shown from time to time as some specific activity, for example of the locomotor apparatus. In this case it is usual to speak of "spontaneous" movements.

It is also possible that this c.n.s. activity, constantly maintained by afferent impulses, may partially, after appropriate transformation, reach a peripheral effector (a muscle, for example) in the form of a constant, non-specific c.n.s. effect on one or other organ. We have assumed that such a reflection of "spontaneous" c.n.s. activity, if it is expressed in the form of efferent impulses, may be recorded in a peripheral nerve with the aid of a cathode-ray oscillograph.

Using intact frogs held by bandages under conditions of maintained circulation and with an undamaged c.n.s., action potentials were led off from one of the nerve branches entering the gastrocnemius muscle. The sheath of the sciatic nerve proximal to the junction of this branch with the main trunk was removed for a distance of about 1 cm. The branch fibres were isolated from the main trunk and placed on smooth silver electrodes mounted on a wide ebonite plate. After removing the fluid from this plate the nerve and electrodes were covered in water-free petroleum jelly.

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By maintaining the connexion of the nerve fibre bundle with the muscle, we were able to record both centrifugal impulses and impulses from the various receptors in the muscle.

The action potentials were recorded by means of a two-channel oscillograph (type OB-2 of the experimental factory of the Academy of Medical Sciences), both amplifiers of which were compensated. The frequency filter range was limited to between 100 and 1500 c/s and in some cases 100–500 c/s, which enabled us to achieve some reduction in the sound level.

During the 4–6 hr of the experiment we observed the continuous "spontaneous" bioelectrical activity in the nerve being investigated, which was shown both as single and multiple deviations (volleys) of electrical potential (Figs. 1, (*a*) and 2). Volleys of impulses were seen in most of the experiments (18 out of 23). These group discharges were repeated continuously at irregular intervals, though the volleys were always synchronous in two symmetrical nerves, as could be seen if their biopotentials were recorded simultaneously (Fig. 2). This fact led to the idea that these discharge volleys were of central origin.

From time to time the animal underwent "generalized movements" [4–7], which in some of the experiments were repeated within fairly short intervals of time. Before the onset of these generalized movements the discharge volleys became markedly more frequent (from 3–5 to 8–10 volleys per 10 sec) and turned into a continuous impulsation lasting for 1–5 sec (Fig. 1(*b*)). The biocurrents in the nerve branch during movements of the animal were shown on the oscillograph as deviations in potential of very great amplitude (Fig. 1(*c*, *d*)). At such a time there is probably both strengthening of the effector impulsations and an "overflow" of the muscle action currents into the electrode leads. Immediately after the end of the movements of the animal, the impulse volleys ceased entirely for 5–15 sec (Fig. 1(*d*)), after which the whole cycle was repeated up to the next generalized movements by the animal.

It should be noted that neither in the period of volley activity nor during the few seconds preceding the generalized movements were we able to record action potentials in the gastrocnemius muscle or to note any contraction or fibrillation of it. We only noted any action currents from the gastrocnemius muscle when it was visibly contracting during the period of generalized movements.

The impulse volleys which we described disappeared after transection of the nerve proximal to the electrodes and were maintained after transection distal to them.

These facts hence leave no doubt that the impulse volleys which we detected were of central origin and, apparently, in the absence of generalized movements by the animal were not accompanied by any excitatory impulses to the innervated muscle.

It is possible that the efferent impulses comprising the volleys, or at least some of the impulses, maintain the tonus of the gastrocnemius muscle [8], but the action currents reflecting tonic contraction of the muscle were not detected, due to their rare and asynchronal rhythm. It is very probable that the impulse volleys represent trophic factors arriving by sympathetic or somatic fibres. Such trophic influences as the impulse volleys (especially when they change to a continuous activity, although still "sub-threshold" for the muscle fibres) may act as a factor preparing the muscle fibres for motor ("trigger") impulses. In the light of new findings on the "spontaneous" activity of the nerve–muscle synapse mechanism [9] it is easy to understand

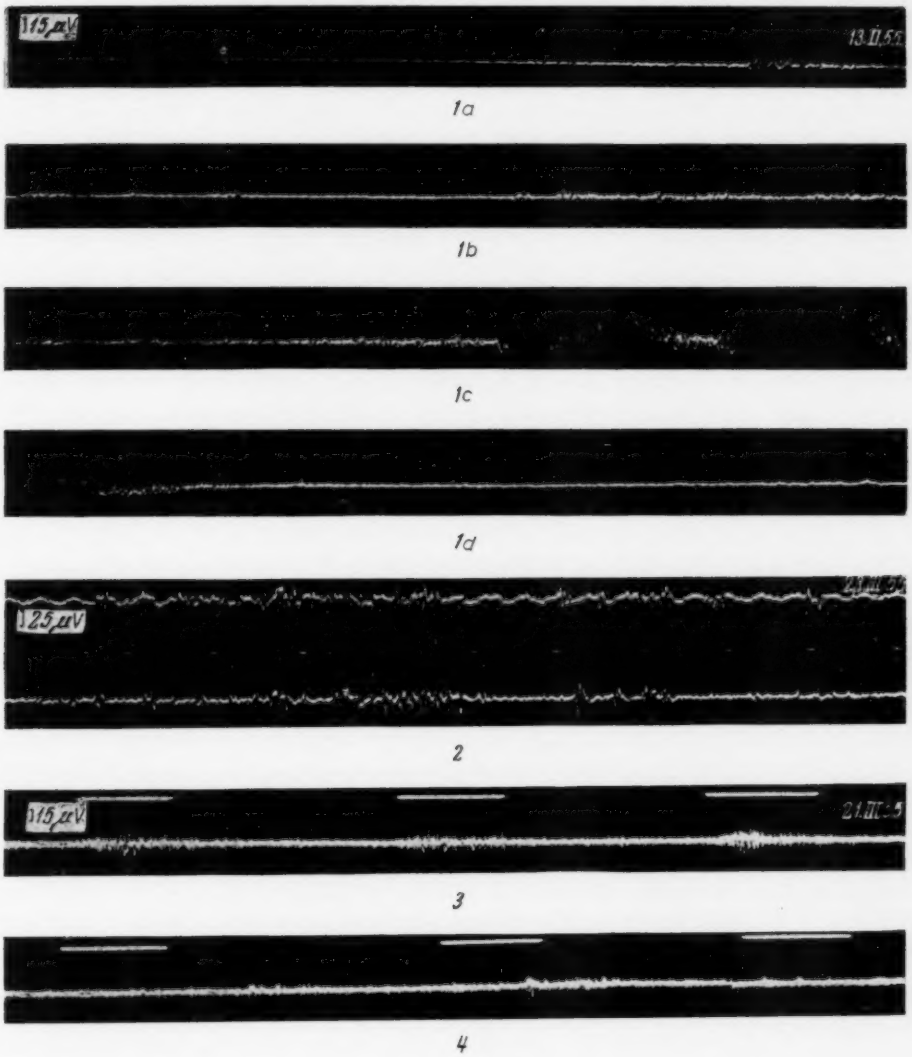

FIG. 1. Oscillograms *a–d*: cycle of changes in the bioelectrical activity of the studied nerve relating to generalized movements by the animal. Oscillogram *d*—proprioceptive impulses are visible at the end of the motor discharge.

FIG. 2. Synchronization of the volleys in two symmetrical nerves; the separate deviations in bioelectrical potential and their phases can be seen more clearly.

FIG. 3. Bursts of impulsation volleys in response to moving the hand towards the head of the frog.

FIG. 4. The effect of the same stimulus; the animal's head was covered by black paper; only "spontaneous" volleys can be seen. Time signals—dotted line (0·05 sec); stimulation signals—thick line over the time signals.

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how important these centrifugal "sub-threshold" influences are to the transmission apparatus of skeletal muscles in maintaining it in constant readiness for activity.

"Spontaneous" discharge volleys, with a rhythm similar to that of respiration, very probably arise under the influence of the inherent rhythm of the respiratory centre by stimuli which are possibly distributed via the reticular formations of the medulla oblongata, the mid-brain and intermediate parts of the brain. The activity may involve secondarily the motor components of the brain, which carry the "spontaneous" impulses to the periphery.

It may be expected that such biologically important stimuli as visual stimulations will influence the "spontaneous" excitations which arise periodically in the brain. Movement of a hand towards the head of the frog did in fact give rise to a volley of impulses of considerable amplitude in the nerve being examined (Fig. 3), which was sometimes followed by contraction of the gastrocnemius muscle. By repeatedly moving a hand towards the eyes it is possible to get a replacement of the "spontaneous" rhythm of the volleys by the rhythm of the optical stimuli. The impulse volleys here quickly weaken in their summated amplitude, in the number of impulses they involve, and in duration, and do not cause contraction of the muscle. Hence, the rhythm of the specific optical stimulation may temporarily predominate over the periodicity of the "spontaneous" excitations. When the eyes of the animal were screened by a piece of black paper this phenomenon did not occur (Fig. 4).

Rhythmical flashes of light in front of the animal's eyes in an otherwise dark environment sometimes also induced discharge volleys in the nerve, with a periodicity approximating that of the light flashes, though to a lesser degree. In other words, "subordination of the rhythm" was incomplete in this case.

All this leads us to consider that the "spontaneous" discharge volleys recorded in the nerve trunks arise and develop in the brain. Additional proof of this lies in the fact that separation of the spinal cord from the medulla leads to the complete cessation of impulse volleys. Ether narcosis has the same effect.

Afferent impulses can also be isolated in the "spontaneous" impulse activity which we observed. This is achieved by transecting the nerve branch proximal to the electrodes, stimulating the muscle and causing proprioceptive impulses, which we equated with "spontaneous" afferent impulses: various forms of impulses were also compared. With the electrodes in the same position in all the experiments, the potential deviations representing the centrifugal impulses had three phases, of which the one with the greatest amplitude (the second one) was directed downward (see Fig. 2). With the centripetal impulses the reverse was the case—the greatest deviation was directed upward (Fig. 2). The relationship was confirmed by a thorough check.

The afferent impulses observed without any special stimulation of the muscle were not grouped in volleys and took the form of a fairly infrequent and arhythmic series of action potentials.

SUMMARY

"Spontaneous" action currents were studied in one of the branches of the sciatic nerve of a whole frog with an intact central nervous system. The connexions of the branch both with the muscle and with the c.n.s. were preserved. "Spontaneous" volleys of centrifugal impulses followed one after the other, more often becoming an uninterrupted impulsation before "generalized movements" by the animal. After

these generalized movements the impulsation ceased for 5–15 sec. Apart from the generalized movements, and without the arising of an uninterrupted impulsation, no action potentials were seen to arise in the corresponding muscle in response to this discharge groups in the nerve. Artificial rhythmical visual stimulations caused the appearance of impulse volleys in the nerve synchronous with the stimulations, whereby the periodicity of the "spontaneous" discharges was replaced by the imposed rhythm.

The results obtained enable us to suppose that the "spontaneous" discharges described arise in the brain under the influence of the activity of the respiratory centre. These excitations are probably propagated via the reticular formation of the brain. Discharge volleys reaching the neuromuscular synapse may act as a factor preparing the synaptic mechanism for the transmission of the proper "trigger" impulses to the muscle fibres.

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AN UPPER OXYGEN LIMIT IN YEAST IRRADIATION LESIONS*

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A STUDY of the oxygen effect, which has been described by many authors, may be of some importance in interpreting the mechanism of radiation effects in organisms.

The effects of oxygen on the radio-sensitivity of chromosomes have been noted during irradiation of the latter [1].

A study of the influence of oxygen on the suppression of growth in irradiated

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bean radicles showed that the presence of oxygen may be looked upon as a factor increasing the dose [2].

It was shown in experiments on new-born mice, which can tolerate complete asphyxia for some time, that during such conditions of oxygen deficiency irradiation with lethal doses does not have a fatal outcome; subsequent development is normal, not differing in any way from the development of non-irradiated mice, whereas control mice, irradiated with the same doses in the presence of oxygen, all died within a few days [3].

Experiments on yeast showed that its radio-sensitivity was increased on irradiation in the presence of oxygen in comparison with yeast irradiated under anaerobic conditions [4].

A quantitative study of the lesions in *Escherichia coli* cells grown in anaerobic conditions before irradiation and irradiated in an atmosphere of nitrogen and cells cultured aerobically and irradiated in an atmosphere of oxygen showed that the radio-sensitivities of the micro-organisms varied in these conditions by a ratio of 1: 10 [5]. An oxygen concentration of less than 1 mg/l. had no effect on the lesions. With higher concentrations, up to 10 mg/l., there is a clear proportional relationship between the amount of free oxygen in the suspension and the percentage of bacterial cells affected. With oxygen concentrations of over 10 mg/l. it is not possible to demonstrate this direct relationship between the amount of oxygen and the percentage of affected cells. Analysis of these results does however reveal that with increasing concentrations of dissolved oxygen the survival curve reaches a minimum and then progresses parallel to the abscissa. The "oxygen limit" which occurs in this case may be considered as one of the limiting phenomena, well-recognized in physical chemistry, which were discovered by N. N. Semenov's school. This paper is designed as an experimental confirmation of this point of view.

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TECHNIQUE

As a subject for irradiation we used the diploid yeast *Saccharomyces vini*, strain Megri-139-B. The yeast was cultured on a solid nutrient medium: 2% agar in a brewer's wash with a strength of 4° proof at 30°C. The agar was poured into special vessels 2·5 cm in diameter and 4 cm deep, with a ground stopper and an outlet tube for emptying and filling the vessels with oxygen at various pressures. The yeast was incubated under these conditions for 48 hr. By the end of the incubation the number of budding cells varied from 0·2 to 0·3 per cent.

After incubation the yeast had formed a uniform film over the surface of the agar. The air was evacuated from the vessel containing the yeast by means of a vacuum pump and the vessel was filled with oxygen at various pressures. To measure the pressures we used a mercury manometer, which enabled us to measure pressures of from 0 to 760 mm Hg. After being filled with oxygen the tubes were irradiated. The source of radiation was a γ-applicator GUT-400 with a half charge of ^{60}Co, giving a dose rate of 20,000 r/min at a distance of 1 cm from the source. Irradiation was performed on the solid medium and not in suspension; all the yeast cells received an equal dose as they were situated in one plane perpendicular to the radiation. The yeast in our experiments received a total dose of 200,000 r.

According to the survival curve which we obtained, this dose causes the death of

55 per cent of the cells under normal conditions. This percentage of affected cells would normally be caused by a dose of 30,000–50,000 r [6]. This discrepancy between our findings and published results can be explained by the fact that in most of the work dealing with the effects of radiation on micro-organisms the latter were irradiated in suspensions, whereas we carried out the irradiation on a solid nutrient medium with a liquor containing sugar and other easily-oxidized substances, which undoubtedly have a protective effect, thus raising the lethal dose. After irradiation the yeast was washed from the surface of the nutrient medium with sterile distilled water and the resultant suspension diluted 1000 times. This diluted suspension was spread on to microscope slides covered with a thin layer of nutrient agar. The slides were placed in Petri dishes and incubated at 30°C for 20 hr.

After incubation the colonies were counted under the microscope at a magnification of 100 times and the percentage of affected cells calculated. The criterion of undamaged colonies was a rounded shape with smooth, even edges. Colonies with uneven, crenated edges, and also chains of cells, single and paired cells, were considered as having been affected. It may be assumed that the single cells were killed during irradiation, the paired and chain forms 4–5 hr later and the irregular colonies 14–16 hr after the irradiation [6].

This technique, first suggested by Korogodin [6], enables the various times of death of the irradiated cells to be determined with great accuracy.

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EXPERIMENTAL RESULTS

We carried out six series of experiments in which we studied the dynamics of yeast cell lesions at various partial pressures of oxygen. All six experiments show that with increasing partial pressures of oxygen the total percentage of affected cells increases between 100 and 300 mm Hg, reaching a peak at the latter point; on increasing the pressure further it starts to fall, and at 750 mm Hg it has returned almost to the original level (Fig. 1). If we follow the changes in the cells at various oxygen pressures, dividing the cells into groups according to their time of death, it may be seen that the curve showing the cells killed under the irradiation against the changes in oxygen partial pressure has no special features (Fig. 2 (*A*)). In the cells dying a short time later (5–6 hr), the percentage affected increases with the oxygen pressure between 100 and 200 mm Hg, remaining constant at higher oxygen levels (Fig. 2 (*B*)). Yeast cells dying some considerable time after the irradiation (15–16 hr) show a marked increase in the numbers affected at oxygen pressures of from 100 to 300 mm Hg. With further increase in the oxygen pressure, the percentage of affected cells falls sharply (Fig. 2 (*C*)).

Hence, it may be considered that the nature of the overall curve of affected cells is mainly determined by cells which die after a considerable latent period.

DISCUSSION OF EXPERIMENTAL RESULTS

In summing up the above findings, it may be concluded that as the partial pressure of oxygen is increased, the growing percentage of affected cells reaches a limit, beyond which further increase of the oxygen pressure causes no increase in the number of yeast cells dying, but even reduces it. This phenomenon may be termed the "upper oxygen limit" for the developmental processes of radiation lesions, using this term

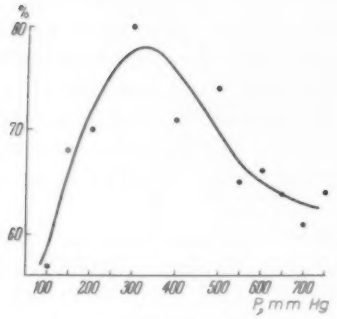

FIG. 1

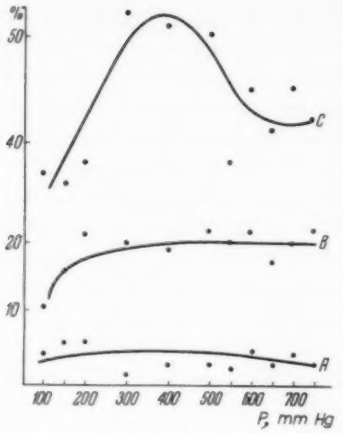

FIG. 2

FIG. 1. Total radiation lesions of the yeast *Saccharomyces vini* against partial pressure of oxygen (P). Dose: 2×10^5 r. Ordinate: percentage of affected cells.

FIG. 2. Death of yeast cells at various times after irradiation against partial pressure of oxygen (P). Dose: 2×10^5 r. Ordinate: percentage of affected cells. A—died during irradiation; B—died 6 hr after irradiation; C—delayed death (16 hr).

in the sense which it acquires from the study of limiting phenomena in the kinetics of oxidation reactions of a chain character carried out in Semenov's school.

We are inclined to consider the results obtained from the point of view of the hypothesis put forward by Tarusov [7] on the chain reaction following the action of an ionizing radiation on a living substrate. On radiolysis of the water in living organisms, free radicals are formed which are very active oxidizing agents. Their output is directly proportional to the amount of free oxygen or its partial pressure.

Semenov's school has shown that in the development of oxidizing chain reactions there is an upper oxygen limit, when the rate of the reaction and its products increase up to a maximum with increasing oxygen pressure, then at higher pressures the reaction is slowed down, finally causing a break in the chain and hence stopping the reaction [8]. As the oxygen pressure increases there is a corresponding output of free radicals. This leads to a possibility of a triple combination, when two radicals come into contact with one molecule of an oxidizing substance. The radicals recombine and the molecule is freed, though not to an extent whereby it is capable of reacting further and initiating another link in the chain, as a result of which the chain is broken and the reaction is slowed down until it finally stops.

We are inclined to suppose that an increased amount of free OH and HO_2 radicals, produced by the ionizing radiations in biological subjects, also leads to this type of combination with molecules of the living substrate, as a result of which the primary oxidation reaction which subsequently kills the organism is depressed. This is reflected as a decrease in the percentage of affected cells when the partial pressure of oxygen is higher than the value corresponding to the maximum. The fact that the upper oxygen limit is observed only in cases of delayed death enables us to suggest that different mechanisms are involved in the different types of death.

CONCLUSIONS

(1) A maximum in the number of cell deaths after lesions caused by the irradiation of yeast cells depends on the O_2 concentration. Increase in oxygen pressure after the maximum has been reached leads to a lowering of the percentage of affected cells. The maximum is seen at an oxygen partial pressure of 300 mm Hg. This phenomenon is similar in nature to that of the upper oxygen limit which is well recognized in physical chemistry.

(2) The phenomenon of an upper oxygen limit expressed as a maximum of cell deaths, with a subsequent decrease in the percentage of dead cells, is only seen in cells whose death has been considerably delayed. The effect could not be demonstrated in cells which were killed during the irradiation or died a short time after it.

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DISTURBANCES IN RETINAL FUNCTION CAUSED BY WEAK IRRADIATION*

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THE state of the visual analyzers of animals or humans subjected to the action of penetrating radiation has an important place in the study of the effects of ionizing radiations. The effects of these radiations on retinal function are being investigated in the biophysics laboratory of the Institute of Physiology, Academy of Sciences of the Armenian SSR [1–3]. The present investigation concerned changes occurring in the retina after the action of small doses of X-rays. We were able to find only a few published papers [4–6] on the study of the condition of the crystalline lens and retina after the action of large doses.

Our experiments were carried out on the isolated frog eye and consisted of two series: in the first, we studied the electroretinogram (e.r.g.) of the eye after removal

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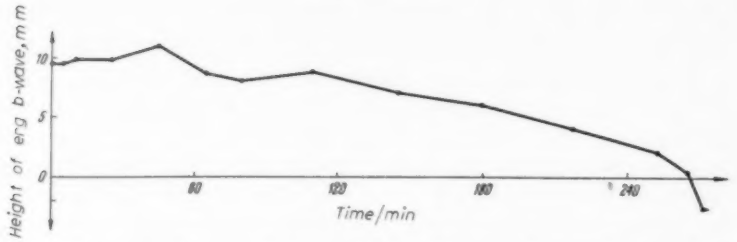

Fig. 1. Changes in the e.r.g. *b*-waves of isolated frog eye in a control experiment.

of the lens and vitreous body, while in the second series the eye was intact. In both cases the e.r.g. was recorded before and for 1–2 hr after the action of the X-rays.

The isolated frog eye or retinal preparation ("eye goblet") was kept inside a moist chamber. Silver electrodes were used to tap off the retinal biocurrents. The biopotentials were recorded on an electrocardiograph with an added photo-relay system

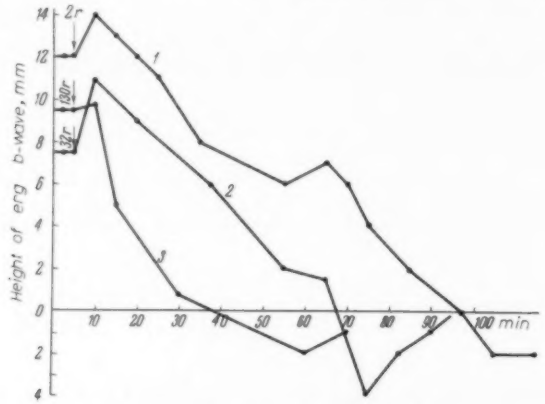

Fig. 2. Changes in the e.r.g. *b*-potential under the influence of X-rays. 1—12 r; 2—32 r; 3—130 r. Irradiation marked by arrow.

for marking the stimuli. The eye was illuminated by a 6V, 21W bulb, the voltage to which was controlled by a voltmeter. The light stimuli were obtained by means of a photoshutter. The irradiation was by X-rays (80 kV, 5 mA, 1 mm Al, focus distance 15 cm). The dose was altered by varying the exposure time. After each cycle of experiments the dose of X-rays received by the test preparation was measured by a GRI dosimeter.

As shown in Fig. 1, in a control experiment the isolated retina will generate biopotentials for 2–3 hr. Ignoring small fluctuations, the control curve falls to the zero line and becomes negative almost without any deviation. Figs. 2 and 3 show the changes in the e.r.g. *b*-waves after irradiating the eye with doses of 12, 32, 75 and 130 r. In most of the cases there was an initial rise and subsequent rapid fall of the e.r.g. curve to zero, with a further fall to a negative value.

In experiments with the intact isolated eye, without removal of the crystalline lens, small doses of irradiation also resulted in an initial increase in amplitude of the *b*-waves, with a subsequent rapid fall of the e.r.g. With greater doses, the initial rise

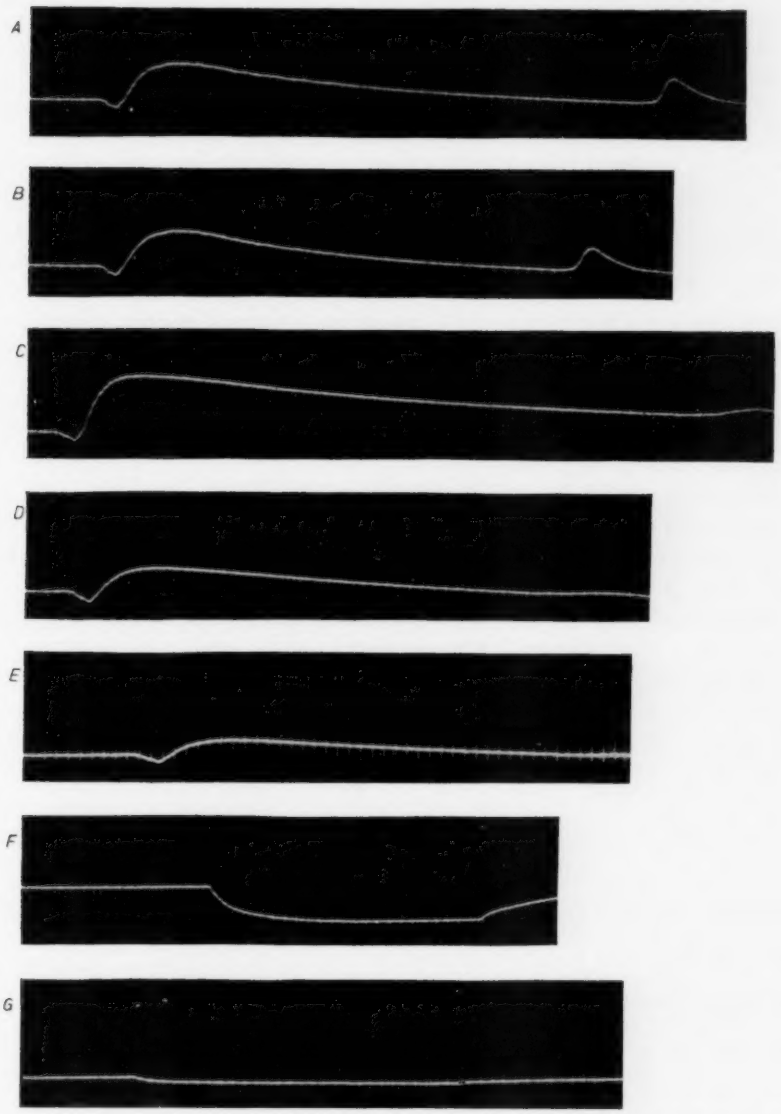

FIG. 3. Increase and subsequent decrease of retinal biocurrents under the influence of X-rays (dose—75 r). A, B—e.r.g. before irradiation; C—phase after irradiation; D—after 5 min; E—after 15 min; F—after 40 min; G—after 60 min. Scale: 5 mm = 250 μV. White dots—start and finish of illumination. Vertical lines—time signals (1/20 sec).

in the e.r.g. does not occur and the curve falls rapidly immediately after the action of the X-rays.

In nearly all the e.r.g.s the fall of the curve to zero is followed by a crossing into the negative phase, and this condition is maintained for a long time—until the preparation becomes dessicated. We consider it probable that this persistent negative

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potential is the result of purely physical processes. Its appearance indicates irreversible death of the retina. It should be noted that a negative e.r.g. potential is seen in other experiments on the retina when its function is irreversibly destroyed. Thus, A. M. Aleksanyan's investigations showed that on increasing the intra-ocular pressure (to over 200 mm Hg) a negative component of the e.r.g. appears, and on subsequent lowering of the pressure the e.r.g. does not return to normal.

Our results, which indicate the considerable changes in the e.r.g. of the frog's eye brought about by weak radiation effects, may be considered in the light of current theories on the biological effects of ionizing radiations. The action of X-rays on a biosubstate gives rise to secondary electrons which, by means of ionization and activation, form active radicals which act upon the visual purple, especially on its protein component containing a sulphydryl group. As a result, oxidation of the —SH group of rhodopsin may occur, with conversion to the —S—S— form. Wold's work has shown the connexion between photochemical processes in the eye and —SH groups, inactivation of which destroys the normal course of these processes and the generation of biopotentials.

In this connexion, it could be supposed that the e.r.g. changes occurring in the living retina under the influence of small doses of an ionizing radiation are linked with the effects of X-rays on the protein part of rhodopsin, which contains sulphydryl groups. Peskin's work should be mentioned here, however [7]; he demonstrated the high resistance of rhodopsin to the action of X-rays. It was found that fading of rhodopsin (i.e. a photochemical reaction) did not occur even with a dose of 5×10^6 r.

We restrict ourselves to the point of view that X-rays have some indirect influence on the functional state of the retina in small doses, but we do not exclude the possibility of a direct effect on the receptor elements of the retina. Whatever the mechanism of this effect may be, the retina is incontrovertibly very sensitive to ionizing radiations and for this reason is an interesting subject for radiobiological investigations.

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EXPERIMENTAL DATA ON THE EFFECTS OF AN ELECTRIC CURRENT OF INDUSTRIAL FREQUENCY AND HIGH TENSION ON THE GALLWORM*

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THE battle against the gallworm (*Meloidogyne marioni* Cornu, 1879)—a dangerous menace in agriculture and landscape gardening—is posing some very real problems. One method of dealing with this nematode in the soil consists (according to Daulton and Stoks [1]) of the use of an electric current of frequency higher than 50 c/s, the opinion being that the effectiveness of this is directly proportional to the frequency of the current, which reaches an optimum value at 250 c/s. These investigators obtained the best results by using a pulsating unidirectional current.

The aim of our experiments was to show the possibility of using an alternating current of industrial frequency (50 c/s) for the suppression of these worms, which would be more expedient in view of the obvious advantages of using a standard current.

The experimental technique in the first series was as follows: 55 cm^3 of damp soil infested with gallworm were placed in a flat container ($3 \times 115 \times 160$ mm) made of an insulating material (glass). The soil was treated by means of two tin electrodes measuring 47×29 mm. The electrodes were fixed to an insulated handle so that it looked like a tuning fork. Flexible leads supplied current from a high-voltage transformer with an increased magnetic scatter (120/13,000V transformer, 150W). Since the electrodes, on being buried in the soil, did not cover the whole width of the container (width of container 115 mm, length of electrodes 47 mm), and the length of the container (160 mm) greatly exceeded the distance between the electrodes (20 mm), the soil was treated in the following manner: the electrodes were put into the soil and drawn through it across the breadth of the container; before the next application of the current the electrodes were moved to the next section, and so on until all the soil in the container had been treated (eight moves). This approximates to the conditions which may be expected in a greenhouse or in the open soil.

Sprouting cucumber seeds (Klinskii variety) were planted in the treated soil; the plants were grown for a month, after which their degree of gallworm infestation was determined. For a control, we grew plants in conditions identical to the experiment but without treating the soil with the electric current.

The electrical tension during each experiment varied within the limits shown in Table 1; these variations were caused by the instability of the electrical charge on the soil. The electrical potential was measured by means of an electrostatic kilovoltmeter.

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TABLE 1. DESTRUCTION OF GALLWORMS BY APPLICATION OF A.C. ELECTRICITY

Expt. No.	Voltage across electrodes (kV)	Average voltage gradient (V/cm)	Time of one application (sec)	Results
1	2–3	1250	22·5	No galls
2	6–7	3250	15	No galls
3	12–15	6750	41·2	No galls

The experimental results are shown in Table 1. The treated soil was heated to a temperature of 70–80°C by the current.

The control plants were in each case severely infested with gallworms. On examining the root systems of the plants grown in the treated soil we found two females and one larva, which had not formed galls, in spite of being in the roots. We are probably dealing here with a suppression of the multiplying capabilities of the gallworms. It is important to note that the cucumbers showed no variation from normal during the time that they were being grown in the treated soil.

The results of the first series of experiments show that at voltage gradients of 1000 V/cm and above the worms are suppressed fairly effectively. This result may, however, be attributed to sterilization of the soil by the high temperature observed during its treatment.

We were interested in explaining the conditions whereby the nematodes are suppressed by a specific traumatic effect of the current. With this aim, we set up a second series of experiments.

In the second series the technique for treating soil infested by the nematodes was somewhat different: instead of a flat container we used glass tubes 45·5 and 63·8 cm long, containing 84·5 and 260 cm^3 of soil respectively. The electrodes were disks of lead foil placed against the soil at the ends of the tube, which also served as stoppers. The source of electric current was the high-voltage transformer of an X-ray apparatus. In some of the experiments the current was switched on and off several times. Control plants were grown in the same conditions but without electrical treatment of the soil. The results of the second series of experiments are given in Table 2.

In all the experiments where infestation was found the root galls could be seen with the naked eye. The control plants were very severely infested. The average number of galls in both control experiments was taken as 100 per cent and the percentage infestation in the electrically treated soil was calculated relative to this.

The last three experiments were performed in conditions ensuring that there was no significant heating of the soil, which meant that in these cases we could assume the predominance of a specific effect of the electric current (electrotrauma). The absence of a heating effect also stabilized electrical conditions in the soil. The electrical gradient sufficient to destroy the nematodes effectively was about 300–500V/cm, with a current density of the order of 0·1A/cm^2 and an exposure time of about 0·5–1·5 sec.

A comparison of the two series of experiments described above makes it obvious that the conditions applying to the last three experiments of the second series (of the

TABLE 2. EFFECT OF ELECTRICAL CONDITIONS AND TEMPERATURE

Expt. No.	Electrical conditions			No. of applications	Time of each application (sec)	Total time of treatment (sec)	Heating of soil (°C)	Volume of soil (cm^3)	Degree of infestation as % of control (100%)
	A	kV	V/cm						
1	0·2	2	31·2	20	1	20	60	260	10
2	0·15	5	79·6	15	1	15	60	260	10
3	0·15	4·8	75·0	1	1·5	1·5	60	260	30
4	0·16	4·5	75·0	1	1·5	1·5	60	260	30
5	0·15	5	78·2	3	2·3	7	60	260	0
6	0·125	15	328·2	1	0·15	0·15	No heating	84·5	1
7	0·125	22	478·2	1	0·5	0·5	No heating	84·5	0
8	0·15	24	521·7	1	1·5	1·5	No heating	84·5	0

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number which we carried out) appear to be optimal in that they ensure complete sterilization of the soil with a very short time of action of the electric current.

CONCLUSIONS

(1) These experiments demonstrate the possibility of using an alternating current of industrial frequency (50 c/s) to combat gallworms.

(2) Besides the effect of heat, we have also noted a specific traumatic effect of the electric current as a result of which the helminths were suppressed.

(3) In the latter case complete suppression of the worms was achieved with a voltage gradient of 300–500V/cm, an exposure of 0·5–1·5 sec and a current density of about 0·1A/cm.^{2}

(4) This work was of an experimental character, but the results obtained may serve as a starting point for the development of an electrical method for combating gallworms.

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A γ-RAY SOURCE (GIBE-800) FOR RADIOBIOLOGICAL EXPERIMENTS*

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Modern radiobiological research covers a very wide field and demands a large amount of technical equipment. Descriptions have been published of a number of specialized installations, developed and constructed to fulfil particular conditions for the irradiation of biological subjects. For example, there are installations [1, 2] which enable doses of thousands of roentgens to be given in a single brief burst lasting 100 μsec (a dose rate of 10^7 r/sec), installations [3] enabling pulses of X-radiation to be obtained which last for only 0·5 μsec, with wide control over the duration and frequency of these impulses, and installations [4] allowing biological objects to be irradiated with huge doses (hundreds of thousands of roentgens) in the course of a few seconds. Also, many important questions on the biological effects of ionizing radiations which call for mass experiments are solved by means of sources which allow relatively prolonged irradiation to be carried out (some minutes) at a comparatively low dose rate (hundreds of roentgens per minute). The construction of reliable installations of this type is therefore very important.

Radiobiological experiments involving irradiation of objects with ionizing radiations must be performed under a number of controlled conditions which ensure that the experiments are set up on a strictly scientific basis. The most important of these requirements, as applied to a source of radiation, are: uniformity with respect to dose of the field where the irradiation takes place, reproducibility of irradiation conditions, the ability to vary the dose rate over a wide range, and the uniform absorption of radiative energy through the whole volume of the irradiated object.

Fig. 1 shows the relationship between the energy (in rads) absorbed by various tissues per roentgen and the energy of radiation. The figure shows that with low-energy radiation the amount of energy absorbed by different tissues varies for any given dose, measured in roentgens [5]. Only with an energy of radiation of 250–300 keV and above does the absorption by different tissues become about equal. For experiments on biological subjects with tissues of differing densities and chemical composition, and also for the irradiation of subjects of considerable size, a source of fairly high energy is essential.

A standard X-ray apparatus working at 200 kV (giving an effective energy of radiation of about 120–140 keV) is not really suitable for this purpose.

SPECIFICATIONS OF THE GIBE-800 INSTALLATION

Taking the above-mentioned requirements into consideration, we chose radioactive cobalt as our source of radiation. To satisfy the rest of the requirements, the

* Biofizika **3**: No. 1, 118–122, 1958 [Reprint Order No. BIO 118].

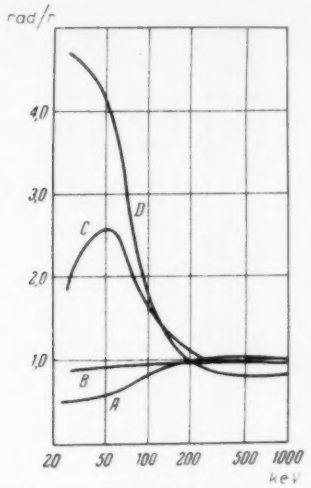

FIG. 1. Relationship between energy absorbed (in rads) per roentgen and photon energy for various tissues. *A*—fat; *B*—muscle; *C*—soft bone tissue; *D*—bone.

source was made in the shape of a hollow cylinder consisting of eight linear sources situated around its periphery. The length of the source is 40 cm, and the diameter of the cylindrical emitter is adjustable. All eight linear sources are moved simultaneously. This allows adjustment of the dose rate and of the size of the uniform dosage field to within the required limits. The long half-life of ^{60}Co and the accuracy with which the diameter of the source can be adjusted (to within 2 mm) ensures that the conditions of irradiation can be reproduced satisfactorily. Fig. 2 shows a schematic section of the installation. The emitter is situated in a well, filled with water, 3·5 m deep and 1·5 m in diameter. Objects in an hermetically-sealed chamber are lowered into the water by means of a vertical shaft. The centre of the chamber coincides with the centre of the cylindrical source to an accuracy of ± 1 cm.

For experiments with animals there are arrangements for ventilating the chamber during irradiation and it is possible to measure biopotentials by means of well-insulated electrical leads.

Each of the eight linear sources consists of 20 preparations of No. 7 ^{60}Co, situated in a brass tube. Aluminium cylinders between the preparations ensure their uniform distribution along the linear source. The total activity of the ^{60}Co preparations is 800 g equivs. Ra. The brass tube containing the preparations is inside a wide duralumin tube fitted with a lid. The duralumin tube stands in a special holder which is fixed to a vertically revolving spindle. Radial adjustment of the linear sources is attained by turning these vertical spindles; the latter, which are situated round the periphery of the well, are connected to a common gear mechanism which allows them to be rotated simultaneously and through the same angle. The handle controlling the rotation of the spindles is fitted with a pointer and scale showing the diameter of the cylindrical source.

The mechanism for lifting and lowering the object chamber consists of two telescopic tubes and a winch fitted with an electric motor. The internal movable

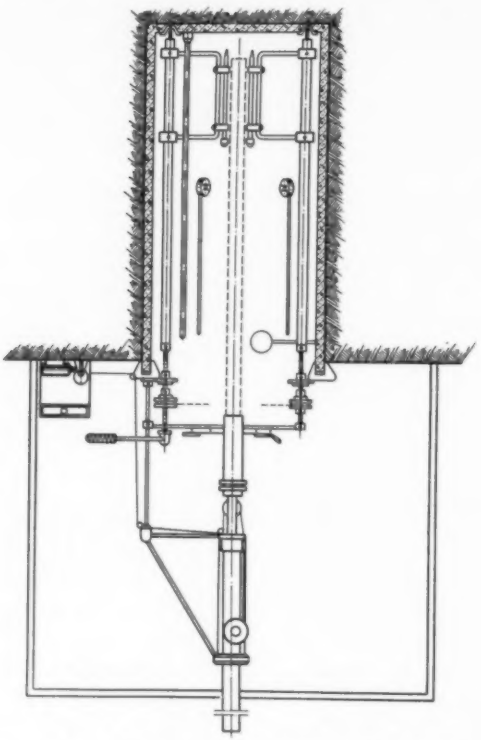

Fig. 2. Schematic section of GIBE-800 installation.

tube is raised and lowered on a cable. The object chamber is fixed to the lower end of the movable tube. Various sizes of chamber can be used. The whole well is covered with a lid, with ports for the chamber and shaft and two observation windows. The inside of the well is lit by two bulbs lowered beneath the water.

The control panel of the installation is situated close to the well. It incorporates the winch, electric motor, the magnetic relays for reversing the motor and other electrical equipment. The panel bears the control switches for the installation itself and for its auxiliary equipment (air supply for ventilating the object chamber, water supply), light signals and electric clocks. The electrical control system provides for the automatic return of the shaft and object chamber to its starting position after the exposure time has elapsed, controlled by an electric clock.

The well is filled with mains water. Automatic maintenance of the correct water level is incorporated. If necessary, the water in the well can be changed in 2 hr by means of a special system of pumps.

Charging of the installation is carried out in the following manner. A special grid 1 m square is lowered into the water to a depth of 1·5 m. The ^{60}Co preparations are loaded on to this from their transport containers with special remote manipulators. The brass tubes of the linear sources are loaded with the preparations and aluminium cylinders and placed in turn into their duralumin jackets, which are then fixed in their holders.

THE RADIATION FIELD OF THE INSTALLATION

The radiation field inside a hollow cylindrical source has a characteristic contour: from the centre to the ends along the axis of the cylinder the intensity falls, and from the centre to the periphery along the radius the intensity rises. There is a uniform radiation field for a considerable part of the volume of the centre of the cylinder.

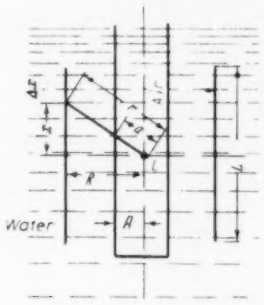

FIG. 3. Schematic section of emitter with chamber inside (to calculate the dosage field in the chamber).

Calculation of the dose without taking into account the weakening of the radiation by the water is comparatively simple, but inaccurate. Calculations taking it into account involve complicated integration and are extremely tedious. The variation of dose within the installation was therefore studied experimentally and the value of the dose was calculated only at the central point of the emitter for any diameter of the latter; it agreed satisfactorily with that obtained by experiment.

Fig. 3 is a schematic section of the emitter with the chamber inside it. Radiations from various parts of the linear sources travel in water for a distance of $r-a$. In Fig. 3, R = radius of cylindrical source, A = radius of chamber, L = length of linear sources.

The path of the radiation in the water will be

$$r-a = (1-A/R)(x^2+R^2)^{\frac{1}{2}}.$$

For the central point of the cylinder and the eight linear sources the dose rate in r/min will be

$$P = \frac{8q\,E_\gamma}{60}\,2\int_0^{L/2}(x^2+R^2)^{-\frac{1}{2}}\exp\left[-\mu(1-A/R)(x^2+R^2)^{\frac{1}{2}}\right]dx$$

where q = the overall linear activity of the source = 2650 mg equivs. Ra; E_γ the "k-factor" = 8·4 r/hr mc cm. The result of the calculation for A = 4·5 cm, L=40 cm and μ = 0·04 cm^{-1} in comparison with experimental findings is shown in Fig. 4.

It was difficult to measure the dose by means of a "Sosna" or "Victoria" type dosimeter, since the integral doses which can be measured in this way do not exceed 50 r. In this connexion it was necessary to prepare an ionization chamber with a small volume and a capacity sufficiently high to measure integral doses of the order of 200–300 r. Teflon was used as the insulating material, since its resistance is not

affected by powerful γ-irradiation and it is not very sensitive to increased atmospheric humidity. Calibration of the ionization chamber was carried out using a ^{60}Co preparation with an activity of 30 g equivs. Ra.

The dose rate at the centre of the irradiated object in relation to the diameter of the cylindrical source (see Fig. 4) coincided closely with the theoretical calculations.

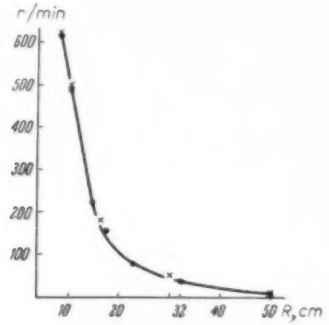

Fig. 4. Dose rate (P) at the centre of the chamber, against radius of emitter (R). Points—experimental, crosses—calculation.

Further measurements showed that with a chamber of radius $A = 4{\cdot}5$ cm and an emitter diameter of 17 cm the dose rate only increases by 5 per cent from the centre to the periphery of the chamber. The volume for which the dose rate remains constant to an accuracy of ± 5 per cent with the smallest diameter of the cylindrical source (17 cm), i.e. at the highest dose rate, corresponds to a cylinder 25 cm long and 9 cm wide.

The installation is also provided with a second chamber, 30 cm in diameter and 30 cm in length.

When the tubes are rotated outwards to a diameter of 41 cm the dose inside this large chamber varies within the limits of ± 10 per cent with a dose rate of 160 r/min at the centre of the chamber.

The distribution of the dose rate in the irradiated volume was also checked by means of chemical dosimetry. The data obtained from these experiments coincided closely with the results of the dosimetry carried out by the ionization method.

The radiational and constructional characteristics of this cobalt installation make it possible to irradiate widely varied objects. With the maximum dose rate (600 r/min in the small chamber) the installation can be used to irradiate potatoes, the seeds of various plants, micro-organisms, mice, rats, etc. Irradiation of larger animals such as rabbits can be carried out in the large chamber with a diameter of 30 cm. The dosage field in this case has a strength of 160 r/min. Still larger chambers may be used, but with a correspondingly decreased dose rate. Finally, it is possible to investigate the influence of the dose rate on radiation effects over a wide range of variations in its value (100 times).

SUMMARY

A γ-ray source (GIBE-800) is described, being the first one to allow the dose rate to be altered over a wide range and to permit the irradiation of biological subjects of various dimensions under optimal conditions.

Complete safety of the installation is ensured, without the use of scarce materials and complicated, expensive equipment. The irradiation process is automatically controlled by means of a time relay, and the simplicity and reliability of the moving parts means that there is a high degree of reproducibility of experimental conditions.

The installation has proved to be quite adequate in work on the experimental irradiation of various biological subjects and can be fully recommended for use in radiobiological research.

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AN ELECTRON MICROSCOPE STUDY OF THE MORPHOLOGY OF THE PIGEON-POX VIRUS*

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(*Received* 29 *April* 1957)

MUCH work has been devoted to the morphology of the pox viruses. As far back as 1941, it was established that the elementary bodies of pox viruses are brick-shaped (Ruska, quoted by Draganov [1]). Many other authors [2–4] have since described round or oval particles and also bodies with a "central convexity". On treating virus particles with pepsin and ribonuclease some authors [5] found that the elementary body has a "nucleus and cytoplasm" and a membrane. Peters and Nasemann [6, 7] stated that the bodies undergo varying degrees of hydrolysis during this treatment. They saw this as proof of the polymorphism of the virus particles. Peters [8] showed that the elementary body has a double protein membrane and a flat rectangular nucleus containing DNA and protein. Two protein bodies are situated in the central part above and below the nucleus. Hertzberg *et al.* [9] and Draganov [1] found pox particles with a low electro-optical density resembling "platelets" and "capsules". Green *et al.* [10] found five dense granules situated at the angles of the bodies in a vaccine. This observation has received no published confirmation.

Important information on the structure of pox viruses has been provided by the examination of ultra-thin sections. Flewwet [11] found that in deep layers of a cell affected by pox virus "foci of vegetative multiplication"—accumulations of oval bodies with a thin membrane in a homogeneous or granular material—developed some 4 hr

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after infection, and after 10 hr immature and mature virus forms are found in the cell. Morgan and Wyckoff [12] found foci of proliferating epithelium with vacuoles bursting through the walls of the cells during the first 1–2 days of pox infection. The vacuoles contained granules resembling elementary bodies. Several authors [13–15] have found a granular or amorphous material containing indistinctly outlined bodies at the centre of the inclusions. Gaylord and Melnick [16] found "development bodies", semi-transparent "hollow" particles, bodies containing a central granule or vacuole and brick-shaped particles in affected cells. It has recently been noted [11, 15] that at certain stages the limiting membranes of the bodies appear to be incomplete. Experimental findings showed that the virus-affected cells contain inclusions, with a granular or homogeneous material containing bodies of varying density, indicating the polymorphic nature of the virus particles.

The present paper relates to a method involving examination of the unrefined virus in order to obtain the best possible pictures. An examination was made of the chorioallantoic membranes of 9 day chick embryos taken 5–7 days after they had been infected with pigeon-pox virus. The given virus had been adapted to developing chick embryos in this Institute [17]. The pox particles were adsorbed on to a collodion film from the native mixture of the virus, with immediate washing and drying [18]. The preparations were contrasted in a low-clearance coating appliance designed by us in conjunction with K. I. Draganov [19]. The examination was performed under an EM-3 electron microscope.

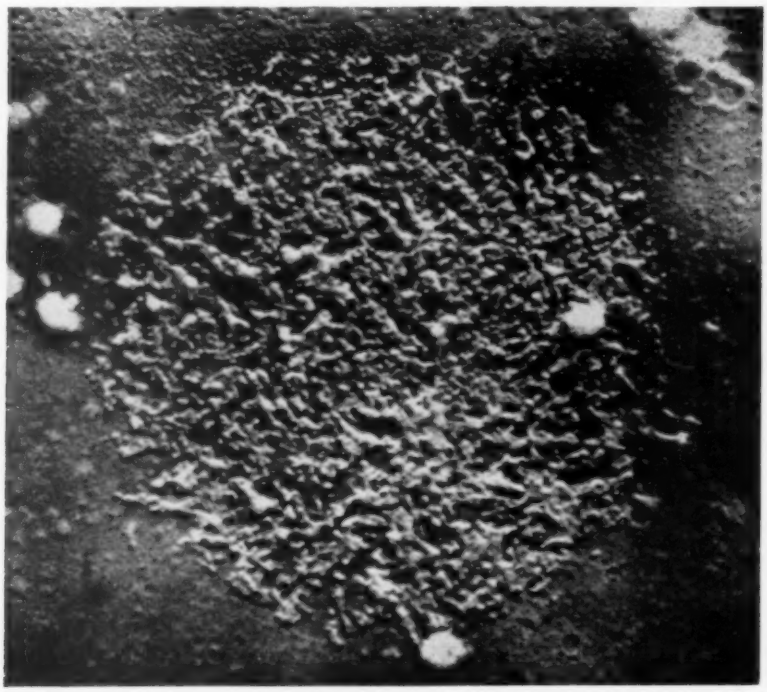

FIG. 1. Intracellular inclusions consisting of granules. Nichrome coated. ×19,000.

In carrying out this work our main attention was directed to the inter-relationships between the various virus particles. The virus was rarely found in slightly affected chorioallantoic membranes (+, ++). With more severely affected membranes (+++) isolated elementary bodies were found, among which were particles resembling "platelets". They were either smooth, with one or two dense, oval granules, or oval, with a granular surface and an incomplete peripheral layer.

In order to avoid possible mistakes in identifying the virus particles, special attention was given to bodies situated within the pox inclusions.

In cases where the infected membranes were severely affected, intracellular inclusions of various dimensions and of a particular structure were found.

By the accepted classification, three types of inclusions could be distinguished. The first of them—the *granular* type—took the form of rounded accumulations of a large number of small, dense, round or oval granules with a diameter of 20–60 mμ. These accumulations were usually not large, but sometimes reached a diameter of 7–13μ (Fig. 1). In the purely granular types such inclusions were rare; more often there was a small number of complete elementary bodies. On further development the granular inclusions were seen to contain larger granules, each of which had at its centre a small number of soft "capsules" with vacuoles, and dense elementary bodies round its edges (Fig. 2).

The second type of inclusion—the *fibrillar*—differed from the first in the presence of a large number of distorted rod-shaped structures, forming a sort of network of interwoven fibrils (Fig. 3). The centres of the inclusions were usually occupied by flat, saucer-shaped, oval or brick-shaped elementary bodies with central vacuoles or convexities.

On studying the rod-shaped structures found in positions more suitable for observation it was established that they are formed from the dense granules described above. For example, aggregations of granules were found in the form of distorted granular rods (Fig. 4). The latter grew thicker and became smooth rods, apparently as the result of the growth of protein molecules. The rods, having reached a certain length, became bent upon themselves to form ovals or rectangular shapes (Fig. 5). In turn, these oval structures formed from the rods became enclosed in a common protein membrane, forming a vacuole which on the drying-out of the particles took the form of a central depression (Figs. 3 and 5). A central convexity subsequently developed at the site of the vacuole. These inclusions were typically enclosed by a membrane.

The third type of inclusion—the *mature* type—was typified by the almost complete disappearance of granular and rod-shaped structures, which had been replaced by flat elementary bodies showing a central convexity and an incomplete dense peripheral layer (Fig. 6). These button-like bodies are flattened, this being well shown when the particles are seen on edge (Fig. 7). There is no membrane round these inclusions; apparently it is disrupted by the growth of the inclusion. Scattered dense complete elementary bodies are seen round the edges of the mature inclusions, containing a few button-like bodies.

Our findings are still insufficient to allow a full interpretation of the complex genesis of pox particles and the whole diversity of their forms. The development of the virus within the cell is apparently linked closely with the cell type and components,

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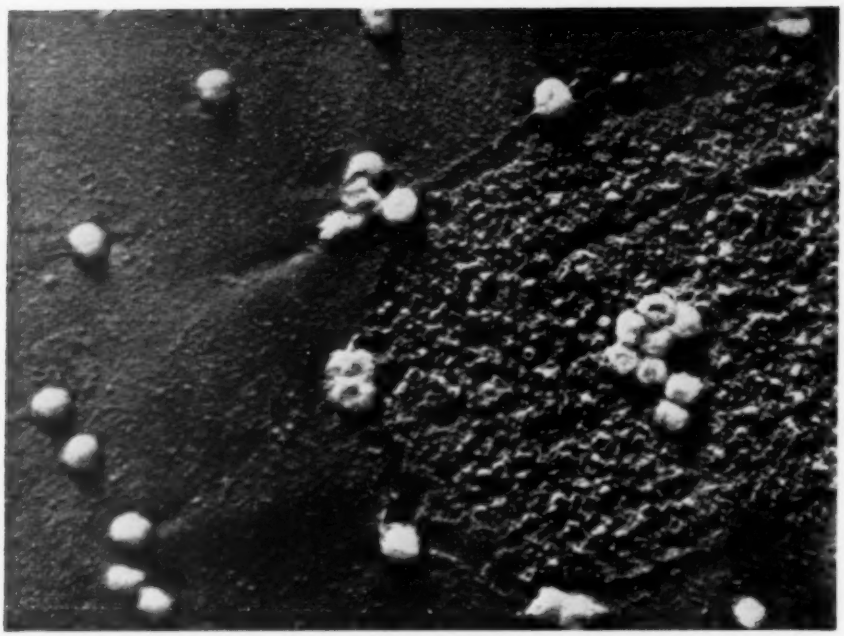

FIG. 2. Granular inclusion. In the centre, soft capsules with vacuoles; at the edges, dense elementary bodies. Nichrome coated. ×21,000.

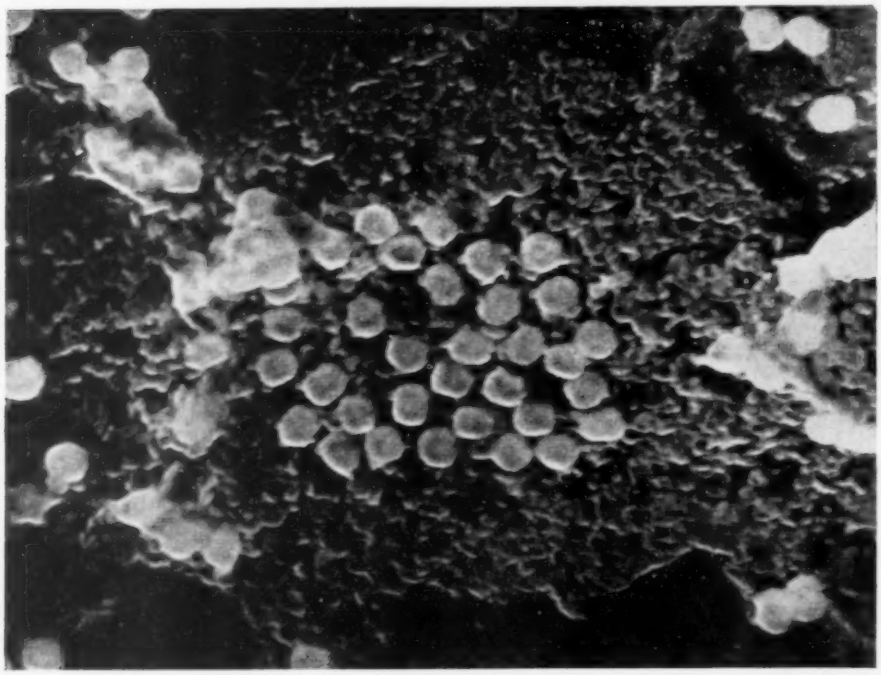

FIG. 3. Fibrillar inclusion. Aggregation of distorted rod-shaped structures; at the centre, soft capsules with vacuoles and developing central convexities. Nichrome coated. ×21,000.

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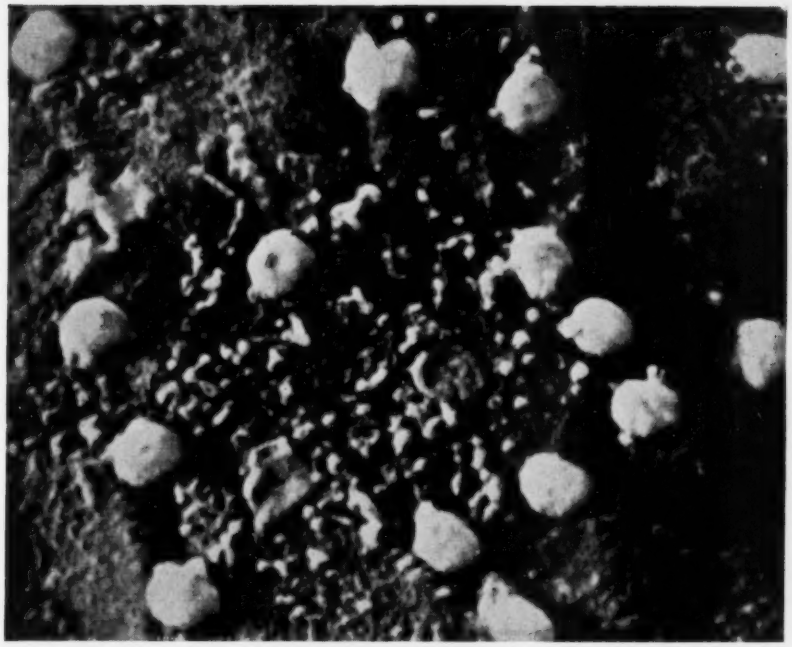

FIG. 4. Aggregation of granules and distorted rods. In the centre, two granular capsules with incomplete peripheral layers. Nichrome coated. ×33,000.

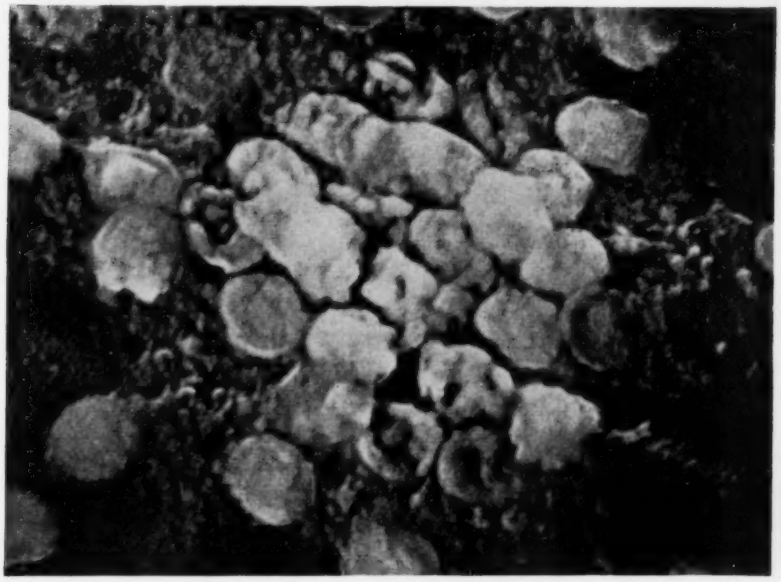

FIG. 5. Aggregation of granules in rod-like structures. Nichrome coated. ×58,000.

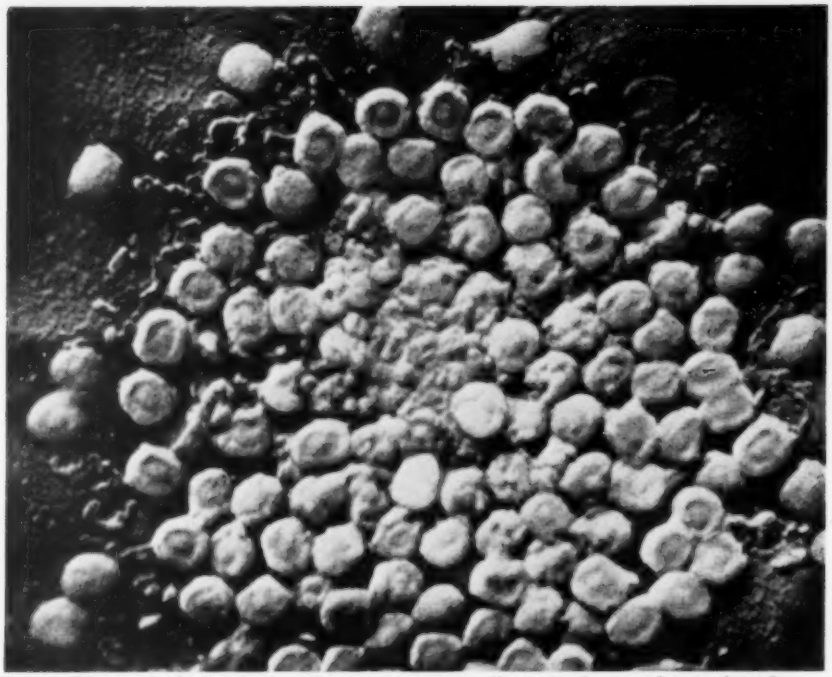

FIG. 6. Mature inclusion body. In the centre, small accumulation of granules; flat, button-like bodies with an incomplete peripheral layer. Nichrome coated. ×33,000.

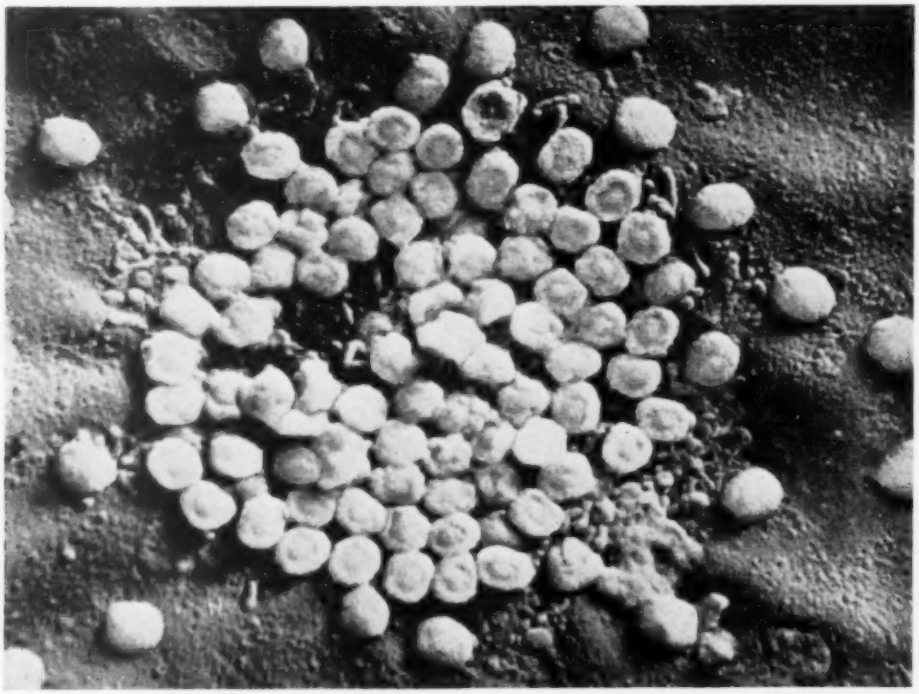

FIG. 7. Mature inclusion body. In the centre, flat, button-like bodies, some of them on their edges. Nichrome coated. ×33,000.

FIG. 8. Diagram of the development of pigeon pox vrus particles. 1, dense granules; 2, aggregations of granules—straight, granular or smooth, rod-shaped structures, closing to form an oval; 3, soft capsules with vacuoles; 4, button-like bodies with a central convexity and an incomplete peripheral layer; 5, dense, complete elementary body.

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as a substrate for the viral development, with its biological characteristics (pathogenicity, virulence, toxicity, etc.), and also with the physiological state of the infected organism.

With regard to the pox virus, Gaylord and Melnick [16] consider that the development of inclusions in pox cases may occur in two ways—from the granular structures through the "hollow" bodies to complete mature particles, and by disintegration of the homogeneous matrix to "hollow" and then mature bodies.

We saw no homogeneous matrix in our material. Five morphological structures were noted in the development inclusions (Fig. 8).

(1) Dense, round or oval granules 20–60 mμ in diameter.

(2) Granular and smooth straight rod-like formations, formed from the granules, becoming horseshoe-shaped and closing almost to form an oval.

(3) Soft, oval vacuolated capsules, 180–200 mμ in diameter.

(4) Flat, oval, button-like bodies with a central convexity, 180–250 mμ in diameter.

(5) Complete, dense, mature elementary bodies.

The rod-like structures, closing to form an oval, which form a dense peripheral layer to the developing particles, can be traced through to the soft capsular and flat button-like bodies. All this enables us to suggest that the particles described above are apparently developmental forms of the pigeon-pox virus.

SUMMARY

(1) During the development of pigeon-pox virus which has been adapted to growth on the chorioallantoic membrane of chick embryos, the cytoplasm of infected cells is seen to contain granular, fibrillar and mature forms of intracellular inclusion bodies (together with dense, complete elementary bodies).

(2) The developing inclusions show small dense granules, rod-like structures, closing to form an oval, soft capsules, flat, button-like and complete, dense elementary bodies, all morphologically related to each other. These particles apparently represent different stages in the development of the pox virus.

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THE STRUCTURES AND PHYSICO-CHEMICAL PROPERTIES OF THE DEOXYRIBOSE NUCLEOPROTEINS*

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Very recently there have appeared data which indicate that the biological activities of the nucleic acids depend to a large extent on their structures, which are determined by their attachment to a protein [1].

In other words, in some cases [2] the active materials are not nucleic acids but nucleoproteins. This is to be expected since in nature the nucleic acids are almost always found combined with protein. While the structures and properties of the nucleic acids have been studied fairly thoroughly, our knowledge of the protein component of the deoxyribose nucleoprotein (DNP) is much less complete, and its structure and properties have been little studied. Even in Chargaff and Davidson's fundamental paper of 1955 [3] there is scarcely any information on the nucleoprotein, the concept even being undefined. Many have so far considered it not as a definite compound with a set of characteristic properties, but of indefinite composition, an artifact produced by the separation [4].

We will consider the characteristics of the various components of DNP. The model of the DNA molecule now usually adopted was proposed by Watson [5–10] and Crick [11–14] from X-ray data; in this the main parts are two coaxial, right-handed, spiral chains of pitch 34 Å and diameter 20 Å. The nitrogen bases lie within the spiral and the phosphate groups outside, a P atom in one spiral falling precisely above a P atom in the other. The nucleotides occur at 3·4 Å intervals along the spiral, the angle between corresponding nucleotides being 36°, the repeat period being 34 Å, i.e. 10 nucleotides. The planes of the bases lie normal to the fibre axis and are joined in pairs by hydrogen bonds. Stereochemical data are taken to indicate [15–21] that hydrogen bonds can only form between adenine–thymine and guanine–cytosine pairs (although it is now claimed that other bases occur in DNA, their presence does not affect the above structure) [15]. The spiral DNA molecules can take up two configurations, a compact crystal *A* and a readily extensible paracrystalline structure *B*. Since only the *B* form can combine with the protein [22] only this form will in future be considered.

Until recently it was assumed that the DNA molecule was a giant double spiral of molecular weight $5–12 \times 10^6$, as determined from light-scattering and viscosity measurements [23–40]. (The values found by diffusion and sedimentation are somewhat lower but are less reliable, because there are major difficulties of principle arising from the large variations in the sedimentation and diffusion constants with

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concentration [41, 42]. Data have now accumulated which compel us to revise our point of view somewhat.

DNA molecules are not continuous, each spiral having breaks between nucleotides which do not, however, come opposite the breaks in the other DNA molecule but are somewhat displaced. The nucleotides would appear to be joined by nonvalence bonds at the breaks [23, 25, 43–49]. Heat treatment of DNA has been shown [50, 51] to give a molecular weight of about 5×10^6, when taken in conjunction with data from forward- and back-titration [21, 52, 53], binding of cationic dyes [54], reaction with Mg^{++} and measurement of the H^+ liberated [55, 56], enzymatic hydrolysis [57] and the actions of ionizing radiations and the incorporation of ^{32}P [44, 47]; the number of breaks then being at least 100–500. Some of these breaks must undoubtedly be due to enzymatic or chemical damage during extraction; dissociation and thermal degradation results show that DNA breaks up, without rupturing ester links, into units of mol. wt. about $15–20 \times 10^3$ [23].

The results led Deccer *et al.* [23] and Sadron *et al.* [25] to conclude that the DNA molecule consists of numerous small units (we might call them submolecules) not bound by valence bonds, but by hydrogen bonds. They are the same for DNA from any given source, no matter how they are extracted, but vary between sources. The following arguments also indicate that these ideas are valid. The DNA molecule, like that of any polymer, contains segments which behave as independent kinetic units; indirect evidence leads us to suppose these segments to have molecular weights of about $30–40 \times 10^3$ [24, 46, 58–63] and to contain two DNA sections (one from each spiral) each of mol. wt. $15–20 \times 10^3$.

DNA is produced by nucleotide polycondensation—it is a polynucleotide; but high-polymer theory indicates that polycondensation of units of weight $30–35 \times 10^3$ is much hindered because certain destructive processes tend to remove the longest chains. We thus consider that the molecular weights of polynucleotides and polypeptides formed by direct chemical reaction (not aggregation) cannot exceed $30–35 \times 10^3$. If we extend Lindstrøm-Lang's ideas on protein structures (given in reference [64]) to DNA we can imagine it built up in the same sort of sequence, as below:

(*A*) primary structure: a chain spiral of valence-bonded nucleotides, of mol. wt. about $15–20 \times 10^3$;

(*B*) secondary structure: a two-spiral macromolecule, built up from submolecules, having breaks along the main chains in both spirals and a mol. wt. of about $5–12 \times 10^6$;

(*C*) tertiary structure: a specifically arrayed double spiral.

The breaks along the chains in the secondary structure facilitate the formation of the tertiary structure. Comparison of data on the dimensions of DNA molecules and of biological structures [38, 65, 66] indicates that the DNA molecule is some 30,000 Å long (mol. wt. 6×10^6), being much longer than the structures in which it is found. It is thus concluded that the DNA molecules can only be of this size if there are points on the molecule opposite breaks where the chain is twisted round. The equilibrium configuration is decided by the relation between the extending forces (mostly due to the high negative charges on the PO^- groups) and those of Van der Waals or hydrogen bond type, which tend to cause the molecule to contract

and the entropy to increase as the limiting statistically coiled form is approached.

The sugar–base bond is stated [24, 67, 68] to be single, free rotation about it being possible, but as this bond does not lie in the main valence chain it can hardly produce large configurational changes. Rotation must clearly occur about some bonds in the DNA skeleton [69]; this is not free, and present ideas [70] indicate that the barrier opposing rotation is several kcal/mole high, the chain tending to the configuration which minimizes the potential opposing rotation. Thus if the configuration does not conform to these minima (as in DNA) the internal forces (e.g. from hydrogen bonds) must be large enough to balance out the potential energy increase associated with rotational distortion [71]. The molecular segments must clearly then be quite large; as the breaks are the natural boundaries between segments the latter must be stabilized by —PO—H— type bonds and residual valence forces [9, 72–74]. This all gives the tertiary structure considerable rigidity, which is overcome, however, when the stabilizing bonds are ruptured by configurational changes. Our conclusions are in agreement with those of many others [27, 75–80]; they are, that the DNA molecule can be deformed slightly, but much less so than in typical polyelectrolytes. The charged PO^- groups lie along the main chain, while in polyelectrolytes they mostly lie on side chains, where the charged groups can rotate more freely; such structures would naturally be more flexible.

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This view conflicts with two extreme views, one that the DNA molecule is absolutely rigid [31, 32, 81–91], and the other that it is fairly flexible [24, 46, 37, 92, 93]. We consider these contradictory data and views to exist because the DNA preparations are not standardized. It has also been clearly demonstrated [94] that the lability and tertiary structure of DNA vary not only with the preparation method, but also with time after preparation.

Thus both the secondary and tertiary structures in DNA are rendered highly rigid by the hydrogen bonds between the chains and stabilization at the breaks; but the primary structure is flexible, as in normal polyelectrolytes [77, 95]. We would therefore naturally expect that any agent which ruptures the nonvalence bonds at the breaks would produce configuration (tertiary structure) changes. In fact, very varied agents, if they do not depolymerize the DNA, produce quite marked contractions [26, 27, 34, 37, 38, 95–112] of some 40–60 per cent in most cases. In some cases the result can be interpreted as due to the DNA being heterogeneous and aggregating, rather than to its contracting, but studies on monolayers of DNA [113] and DNP [114], and the effect of DNA on protein monolayers [115] indicate that the DNA molecule can contract.

If the bonds which stabilize the breaks are ruptured the molecule should become more flexible; since the negative charges are closely packed the first effect of bond rupture should be to extend the molecule. In actual fact a slight rise in viscosity, followed by a fall, is found on treating DNA with molar choline chloride [100], the same effect being also found with denaturants at low concentrations [105, 112, 159]. The viscosity drop would appear to be explained by blocking or neutralization of the phosphoryl groups, leading to contraction (increase in entropy); in the third stage of this process the stabilizing bonds would be restored in the altered structure, a new rigid tertiary structure resulting.

Since DNA reacts with a protein to form DNP this protein must be the protein

component of DNP; unfortunately its structure and composition have been much less studied than those of DNA. The results obtained on the characteristics of the DNP protein by different workers are contradictory, probably because, on the one hand, different preparative methods are used, and on the other, the protein mixture found in tissue has similar properties, slight changes in technique then giving different compositions for the DNP protein [116]. With any of the current preparative techniques we almost certainly obtain a mixture of nucleoproteins consisting of the nucleic acids and proteins of differing chemical compositions. Although much has been published on the fractionation of nucleic acids and DNP no separation of DNP into individual fractions has so far been effected. The DNA and protein may also reassociate during the separation; in fact everything points to the DNP (particularly the protein part) being heterogeneous.

But some reliable facts on the DNP protein are available, in spite of the obscurities and contradictions. We shall examine these in somewhat more detail. DNP is known to contain basic proteins of protamine and histone types, but some 7–9 per cent of the protein is much more firmly bound to the DNA than the rest. This residual protein is not extractable with dilute acids and does not split off in M NaCl [65, 117–121]; it has been detected in various ways, some having determined it directly on the DNP [117, 122, 123] while others have detected it in the nucleus [124–127]. That separated from the nucleus has been fairly thoroughly studied [116]; it is a lipoprotein (8–9 per cent lipid) with an isoelectric point at pH 4·5–5·0 (hence termed acid protein): it may possibly contain carbohydrate residues [128, 129]. But the residual protein prepared from the nucleus and from DNP cannot be equated, as is sometimes done, although they both contain tryptophane, unlike the histones. The nuclear species is bound to nucleic acid, being liberated with DNAase, but the identity of the two has not been demonstrated by direct experiment. In future we shall speak only of the DNP residual protein, the nature of which is so far unknown, some supposing it to be an acid protein containing tryptophane, while others find that, although it contains much more S and tryptophane than do histones, it has an alkaline isoelectric point; finally, some consider it to be a normal histone, but one high in arginine [121]. Thus we do not know at present what is the nature of the residual protein, though the amount is fairly reliably known, being some 18–20 per cent of the total DNP protein component.

The readily dissociated or acid-soluble fraction is a mixture of basic proteins of protamine or histone type. Ultracentrifugation and electrophoresis have given fair agreement between workers as to this component; it consists of two histone fractions (of high and low molecular weights) plus an unidentified dialysable fraction [116, 118, 121, 130]. The molecular weights of the fractions are variously given; some give 8400 for the low fraction [131], others $11–16 \times 10^3$ [119, 120], while 18×10^3 and an axial ratio of 28: 1 has been claimed [118]. Values of 37×10^3 and $130–140 \times 10^3$ have been given for the high fraction [119, 120, 131]. The high values given by some are clearly due to molecular aggregation; thus the $16–18 \times 10^3$ histones are clearly due to dimerization from 8×10^3, and $130–140 \times 10^3$ results from four molecules of weight 35×10^3. The high and low fractions also differ in aminoacid composition; chromatography [132] shows the low to be richer in alanine and lysine and poorer in arginine, glutamic acid, leucine, isoleucine and tyrosine, no methionine or cystine

being present, while the high contains these latter, plus phenylalanine and histidine [119, 120, 132]. Certain data indicate tryptophane as present in both [119, 120].

In fractionating DNP it has been found that the first fractions are richer in lysine and the latter in arginine [133], i.e. the fractions are predominately low and high molecular-weight histones respectively. Analysis of thymus DNP shows it to contain 42 per cent DNA and 58 per cent protein, the residual protein being 9 per cent and the histones 49 per cent, divided 31 to 18 between high and low [119, 120]; but more numerous histone fractions have been detected by chromatography. Thus five fractions have been found in DNP histone, of which the first three (*A*, *B* and *C*) comprised 10, 22 and 3 per cent respectively, of the total DNP protein [134, 135]. These fractions have been prepared directly from several organs, and it would appear that *A*, *B* and *C* contain successively less lysine and more argine, in that sequence. It has also been found very recently that the high and low histone fractions are themselves heterogeneous, each consisting of 4–5 proteins of minimum molecular weight $8–10 \times 10^3$ [136].

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It has not yet been determined whether all these histones occur together with one DNA molecule, or whether each has its own specific DNA, so we can say little about the compositions of the individual DNP fractions. However, there is no need for this, as we can discuss the DNA–protein reaction in a general way, while experiments using artificial complexes of DNA and specific proteins can give us the general laws.

As regards the mode of nucleic acid–protein combination, we note that Astbury and Bell [137] in 1938 had already claimed (from X-ray data) that the nucleotide repeat period (3·4 Å) corresponded to the distance between side chains in a fully extended polypeptide chain; they supposed a salt-like electrostatic link between the phosphate groups in the polynucleotide chain and the arginine in the polypeptide one. Up till now these ideas have not in essence been altered; not long ago it was decisively shown [138] that a similar DNP structure exists *in vivo* and is not produced by the separation, as some have supposed.

The state of the protein in the system is as yet unclear; data exist which agree with the idea of Astbury and Bell that the polypeptide chain is fully extended in the β-configuration [65], while it has also been claimed that the chain is only partially extended [139]. Our experimental data, discussed below, indicate that some of the protein is in the β-configuration, in complete steric correspondence with the polynucleotide chain, and exceeds by several times the residual protein, which is partially altered and fairly densely packed. It is also clear that not all the protein in DNP is identically bound; Fleming and Jordan [140], and earlier Belozerskii [122], demonstrated that the bonds to the various proteins were of different strengths. A hypothetical nucleoprotamine structure has been proposed from X-ray data [22, 66].

In this model the protamines are so positioned relative to the DNA that the basic end groups are bound to the phosphate groups in the DNA, the completely extended polypeptide chain surrounding the DNA spiral. The repeat distances along the chain are equivalent to the nucleotide spacings, while the distance between the ends of the side chains direct to the other side is equivalent to the spacing between the two phosphate spirals along the screw axis in the DNA. The polyarginine part of the protamine can combine with the DNA without disturbing its structure, though this

can only occur if the non-basic sections are somehow deflected so the arginine can approach the phosphate. This is only possible if they form an arc between two arginines, which is probable if two occur together. It has been shown that they do in fact occur in pairs in the protamine [22].

It was subsequently found that the DNA molecule has two grooves of different depths, in which the polypeptide chains lie; the above arcs then occur on the side opposed to the main nucleotide spiral [141]. Davison and Butler [121] have recently proposed a nucleohistone model. They first showed that the thymus nucleohistone, prepared by extraction with solutions of low ionic strength was of quite definite fixed composition, some 13–15 per cent of the DNA phosphate groups not being associated with basic histone groups and lying between adjacent protein molecules. The above nucleoprotamine structure was proposed in modified form for nucleohistone; since here the basic aminoacids form about 25 per cent of the total, and if we assume the histone structure regular, we must have three non-basic residues between basic units. If the phosphate groups are to be neutralized by basic residues separated as above, we have two ways of constructing a model:

(1) the histone lies in a zigzag between the phosphates of the two nucleotide chains, the non-basic residues lying in the centre and filling the recesses in the DNA spirals, the spatial configurations then conforming;
(2) the basic groups join on to one spiral and the protein reproduces the DNA spiral; here some 14 per cent of the DNA surface is not covered by the protein.

These two models are the basic ways of fitting together the DNA and polypeptide chains (the former existing as a double spiral) but they give no indication of the macromolecular structure or the properties of DNP. Since the protein is not merely bound to the phosphate via salt-like linkages, but also via alkali-labile hydrogen bonds [3], the DNP molecule has a shape different from that of DNA. We have thus to consider what specific properties of the DNP differentiate it from its components (protein and DNA) and what amount of protein is necessary and sufficient to give the DNP. There is no direct answer to the latter problem in the literature, though much data indicates that we can suppose that the DNP molecule is a fairly well defined unit of protein content about 55–62 per cent and an N: P ratio of about 3·5–4·1 [121, 142, 143]; but we do not know whether this amount is the minimum necessary to give DNP. We can only get a satisfactory answer by analysing the properties of DNP which differ from those of DNA and determining the value of the protein: DNA ratio at which the specific DNP properties appear.

The new DNP properties produced by the combination of protein with DNA are due to changes in the tertiary DNA structure; we have already noted that certain nonspecific agents can effect this, and the same picture is presented by the DNA–protein combination. The major differences between the denaturants and protein are, however, determined by the initial DNA structure changes being decided by the protein structure and configuration, i.e. the tertiary DNP structure resulting from the DNA structure must be very precisely related to the initial configurations of protein and DNA.

The protein joins on to the DNA via the phosphate groups, and also (a very important point) is joined by salt-like or hydrogen bonds to the breaks in the DNA. Since the protein can also block bonds at these breaks it acts as a "plasticizer" to

the DNA, so in both cases the protein renders the DNP somewhat flexible, the flexibility approaching that of single polynucleotide chains, but of larger segment size. The rise in flexibility is due to the PO^- groups being neutralized. It has also been observed several times that DNP shows much more marked highly-elastic properties than does DNA [1, 65, 144–154]. As with the denaturants, the DNA molecule is much shortened by forming DNP (by 40–60 per cent), as the lower asymmetry of DNP compared with DNA shows* [36, 152–157].

The average number of nucleotides between breaks is 80, so in a DNA chain of weight 5×10^6 there will be about 90 breaks; this means that at least 90 protein molecules are required to screen and disrupt the bonds between breaks. The amount of a protein required to change the configuration then depends specifically on its molecular weight; e.g. if the mean molecular weight of a histone fraction is $20–25 \times 10^3$ the amount needed to produce the DNA→DNP transition is 20–30 per cent. If we suppose, as above, that the residual protein determines the DNP structure, its molecular weight can be estimated, being about 12×10^3, if one molecule screens two opposed breaks, and 6000, if one only.

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The protein configuration cannot be predicted under these circumstances, but fully extended β-chains, corresponding sterically precisely to DNA, form about 20–30 per cent by weight of the DNP also, this amount being independent of both type and molecular weight, and probably sufficient to perform the DNA→DNP tertiary structure conversion by providing the requisite elasticity. It is thus clear from these two types of array that the residual protein blocks the breaks and adopts the completely extended β-configuration. Naturally, the amount of protein required to give the DNP configuration, if present as β-chains is less, in molar terms, the higher the molecular weight, although the per cent content will be the same. Large protein molecules will also cover several breaks and thus reduce the highly-elastic deformation, as is actually found [153, 154].

Thus the phosphate group neutralization and break blocking enable the DNA to take up a statistically more probable configuration in DNP, which latter is also highly elastic. The DNP loses water, and while the DNA dissolves well, the DNP does so poorly; in fact DNA and DNP differ in solubility. The tertiary DNP structure is produced via rotations about certain single bonds opposite the breaks in the main valence chain in the DNA. The most important centres which alter when DNA is denatured or combines with protein are those which involve hydrogen or other non-valence bonds, i.e. those which stabilize the breaks in DNA; the phosphate group neutralization here plays a large part. The DNP molecule shows some fresh properties, namely high elasticity, lower molecular asymmetry than DNA, and a different solubility.

With a 20–30 per cent protein content with the completely extended β-configuration the acid groups on the polypeptide chain are directed outwards, i.e. repulsion will occur between identically charged surfaces of DNP molecules, which will clearly

* We noted above that relatively free rotation about N (base)—C (sugar) bonds was possible (internal rotation angle 45°). We may suppose that in DNA the state where the sugar residues in both chains rotate on different sides, whereas in DNP rotation on one side is the more probable. The transition between the tertiary structures of DNA and DNP will be accompanied by a 50 per cent reduction in repeat period and a corresponding viscosity change. This idea agrees with the new model of DNA, as consisting of identical polynucleotide chains, recently proposed [67, 68].

prevent any supra-molecular structure forming. If the protein content is higher its orientation must be less strict; all the phosphate groups in the DNA will also be taken up, so differently charged areas will result on the surface. The water loss also increases with the protein content; both these factors will facilitate the molecules coming closer together and the formation of a supramolecular (fourth-order) structure; this can clearly only occur for protein contents of above 20–30 per cent. But if the protein forms loops on joining on to the DNA (as will be more common the higher the non-basic amino acid content) the fourth-order structure becomes more probable. The more or less constant composition of DNP (N: P = 3·4–4·1) indicates that the amount of protein producing the above structure is roughly constant. It is assumed [65] that the two types of histones (lysine- and arginine-rich respectively) found in DNP may play different parts; the second is joined to the DNA via the arginine while the first is joined to the resulting DNP by residual valencies and produces the gel-like DNP structure. Thus we get a number of specific properties in the DNP which arise from the protein interacting with the tertiary structure in DNA to give the specific tertiary and fourth-order DNP structures.

We now seek to decide the problem mentioned earlier, namely the minimum amount of protein necessary and sufficient to give DNP. Unfortunately there are as yet virtually no data on the properties of DNP in relation to protein content, though Doty *et al.* have found [158] that a two-fold increase in viscosity occurs on completely deproteinizing DNP. We have tried to find the DNP→DNA transition point by measuring the viscosity of stepwise deproteinated DNP; the viscosity does not alter over a wide protein content range (N: P $\approx$ 5 to N: P $\approx$ 2), i.e. the tertiary DNP structure is configurationally stable. But when the protein content falls to 15–20 per cent, a sharp rise of about two-fold occurs, the DNP→DNA tertiary structure transition occurring [153]. Thus some 15–20 per cent protein is the amount required to give the nucleoprotein properties.

Others have reached the same conclusion on the DNP→DNA transition from indirect evidence [72]; thus DNP does not exist below this protein level, but only DNA joined to a certain amount of protein. The amount of residual protein in DNP is of about this order, so the reason for the difficulty in removing it is clear, because the tertiary DNP structure has to be destroyed, which involves doing the same for the tertiary (and even the secondary) DNA structure; hence the statements that the residual protein cannot be completely removed without disrupting the DNA.

The relaxation properties of DNA appear when the long-range order in DNP is produced. These fourth-order structures depend on the DNP concentration as well as on the presence of protein. Our data on nucleoproteins prepared from liver and pancreas [153, 154] showed that at normal DNP concentrations (0·01–0·1 per cent) the relaxation properties appeared at about N: P = 3·8–4·2 (56–62 per cent protein), i.e. at about the same point as in pure DNP, as described in the literature.

Our experiments on artificial complexes between DNA and certain proteins [152, 154] also throw light on how the protein is bound to the DNA and what is the maximum amount which can be bound. Even at the minimum possible DNA content some properties of DNP are unaltered, i.e. even at 5 per cent DNA we cannot consider the DNP as a protein plus DNA impurity. We also find a viscosity step when the DNP is formed in the artificial mixtures, but the changes, although rather similar,

are different; so every protein gives its own specific DNP structure. Comparison of the maximum amounts of the various proteins which can complex with DNA, and which have various basic group contents, shows that the DNA: protein molar ratio depends strictly on the molecular weight of the protein (for a given DNA), the amount bound being inversely proportional to the molecular weight. The weight ratios are constant for the various proteins, which indicates that the areas of the molecules play an important part, as well as the salt links to the phosphate groups.

DNA of molecular weight 5×10^6 can bind up to 1000 molecules of serum albumin or 2900 of α-chymotrypsin per molecule; these numbers cannot be accommodated in either the native or fully extended states; an array with the molecules on axes perpendicular to the DNA axis can be rejected on the grounds of the known molecular asymmetries. We have therefore to suppose that some of the protein is present in the β-form, fully extended, while the rest is present in some special form, different from the native closely packed state. Of course, we can assume that the protein is present as multiple layers, but viscosity and relaxation data contraindicate this, since the viscosity is unaffected by increasing the protein content, so the configuration must be constant. The addition of a single protein monolayer to a DNA molecule should alter the asymmetry of the latter markedly; on the other hand the change in relaxation behaviour on increasing the protein (allowing for the change in viscous behaviour with protein content) indicates that the "centres" responsible for the highly-elastic properties are gradually being blocked. The formation of intra- and inter-molecular bridges clearly plays a large part in the loss of elasticity. With serum albumin at molecular ratios of 900–1000 the recovery of formed DNP is zero, while the viscosity is no different from that at 350–400. This all indicates that every protein molecule is in contact with the DNA, which can only occur in a scale-like structure.

Our experiments thus show that the fourth-order structure in DNP becomes steadily more rigid as the protein content increases, and at 90–95 per cent protein the highly-elastic deformation parameter becomes zero; this can be considered as the protein saturation point, being the third inversion point of DNP and being biologically very important. Thus viruses contain about this amount of protein, which must provide them with a rigid structure, which in all probability acts as a protective layer and preserves their infectivity. As the protein is removed the structural lability and infectivity increase; this illustrates the profound relation between their structural and biological properties.

The above discussion leads us to conclude that DNP has three inversion points: the first at 15–25 per cent protein, the DNP molecule proper appearing here, with its specific tertiary structure and elasticity; the second at N: P = 3·6–4·1 (55–62 per cent protein) where the fourth-order structure appears (over a wide DNP concentration range), this having its typical structural and mechanical properties; and the third at 90–95 per cent protein, which is accompanied by loss of the highly-elastic properties and, possibly, of the biological activity.

The ideas proposed are naturally only working hypotheses deriving from our own experiments and from published data; they are not intended to explain exhaustively all the phenomena related to the formation and properties of DNP.

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THE TEMPERATURE VARIATION IN THE VISCOUS PROPERTIES OF VARIOUS TYPES OF MUSCLE*

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THE fibrous structure of muscle, which results from the linear aggregation of contractile proteins (mostly myosin), determines the mechanical properties of muscle, i.e. its high extensibility, low elastic modulus and relatively high recovery on deformation. The high polymers have somewhat analogous mechanical properties; studies on their temperature variations have shown that temperature rise causes important structural changes, resulting in lowered mechanical parameters [1]. It has also been shown that all such substances only show their highly-elastic properties within defined temperature limits [2]. Temperature is also known to affect the functional state of muscle, the contraction rate being reduced and the latent period increased by cooling, while the reverse occurs on raising the temperature. This all indicates that the contractile properties depend on temperature.

Much experimental data on the effect of temperature on the structural aspects of living protoplasm has now accumulated. The changes occurring on raising the temperature of the most varied living objects are always of the same types, i.e. reduction in degree of dispersion, two-step viscosity changes (an initial fall followed by a rise in the sublethal range), changes in the degree of swelling in the protoplasm [3, 4]. The internal friction (viscosity) is the most commonly used index of structural change on heating.

A series of studies on single fibres directed by Buchthal [5–7] has shown that the mechanical properties vary markedly with temperature, which also indicates a definite structural reordering in the muscle protoplasm. It was found [6] that the viscosity and elasticity gradually fell in the range 0–25°C, the temperature variation in the viscosity being specially emphasized, the elasticity falling 1 per cent/°C, while the viscosity fell 2 per cent/°C. Sten-Knudsen [7] has measured the torsional elasticity of muscle fibres at temperatures from 0 to 19°C and has found that it falls as the temperature rises.

However, studies of the same kind on intact muscle are no less important as the entire complex contractile system is present. It has been found [8] that frog calf-muscle shows a marked rise in viscosity on cooling from room temperature to 0°C, whereas on raising the temperatures (from room) the viscosity falls almost linearly, a kink occurring at 35°C, accompanied by a sudden rise in viscosity. Zhukov [9, 10] has also found evidence from the adductor muscle of the fresh-water mussel that temperature changes affect the viscosity, as would be expected.

Certain published data on the temperature variations of viscosity for muscles and

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polymers indicate that the two differ essentially. Here we give data on the viscosities of various types of muscles over a wide, though still physiological, range; we explain these by comparison with the temperature variations of mechanical properties found in high polymers.

METHODS

We used the damping of torsional oscillations to indicate the internal friction (viscosity) as a function of temperature. The apparatus used is illustrated by Fig. 1.

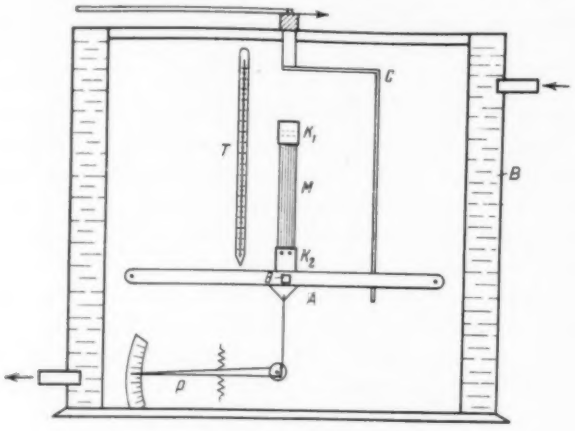

FIG. 1. The apparatus.

The muscle M is fixed in the clamps K_1 and K_2; when the pointer P reads zero the muscle is extended by its own weight and that of the arm A. The changes in extension during the experiment are followed from the movement of P. The muscle can be given various initial extensions by means of the dynamometer lever, the system being set in motion with the coupling rod C, the same amplitude (5°) always being used. The system performs oscillations, recorded by reflecting a beam of light from the mirror R attached to the centre of A, which beam is then passed to a photographic recorder. The apparatus is fitted up in a humidified box B, the walls being heated or cooled (T = thermometer). The logarithmic decrement Q is calculated from the oscillation curve using the formula

$$Q = {}^1/_n \ln (A_{n+1}/A_1) \qquad (1)$$

n being the number of oscillations, A being the amplitude of the first and A_{n+1} that of the last. The following muscles were used:

(1) Frog thigh extensor, straight abdomen, longitudinal and transverse strips from the stomach.

(2) Parallel-fibre strips from *Anadonta* adductor muscle.

RESULTS

On gradually raising the temperature from 0°C or thereabouts Q varied as shown in Figs. 2–7; Figs. 2, 4, 6 and 7 have the chamber air temperature as abscissa and Q as ordinate. Curve 1, Fig. 2, is for frog extensor thigh muscle first cooled to 0°C

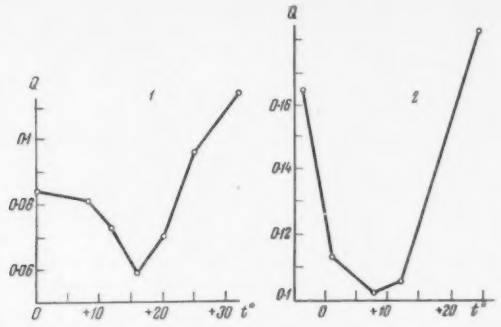

FIG. 2. Relation of log decrement to temperature for frog thigh muscle.

followed by a gradual rise to +32°C; Q initially falls to a minimum at +16°C, and then rises, being 1·3 times larger than at 0°C when +32°C is reached. The same type of curve is found if the initial temperature is taken somewhat below 0°C; sometimes, however, the minimum is not clearly seen. The kink does not always occur at precisely the same temperature, e.g. curve Z of Fig. 2.

In a control experiment (Fig. 3) Q was measured at a constant temperature of +19·5°C; the changes in Q with duration of the experiment were small and indicated that the temperature effects found are not due to the time taken (3–4 hr). Measurements were made every 20–30 min. Similar experiments were performed with straight abdomen from muscle, which is of mixed type, showing both tonic and tetanic properties. Fig. 4 shows where the muscle was heated from 0 to +38°C; at +25°C Q was minimal, the minimum being displaced to the right as compared with

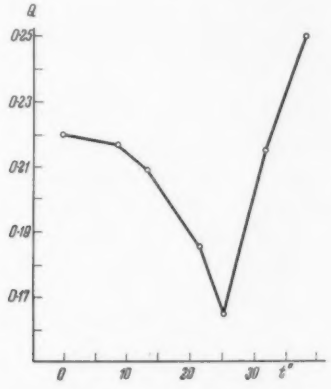

FIG. 3. Relation of log decrement to temperature for frog straight abdominal muscle.

curve 1 of Fig. 2. As before, prolonged control at a fixed temperature of +19°C showed but slight changes in Q (Fig. 5).

In the next series, the relation of Q to temperature was studied on *Anadonta* adductor muscle, curve A of Fig. 6 showing the results for one such experiment. In this case Q fell as the temperature rose from +5 to +22°C, being minimal at +22°C, and then rising, the rise being 20 per cent at +35°C. Smooth frog stomach-wall

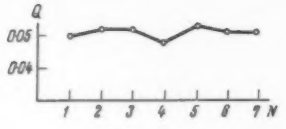

FIG. 4

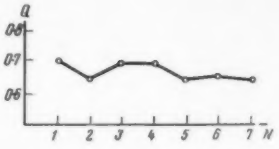

FIG. 5

FIG. 4. Relation of log decrement to duration of experiment at constant temperature (+19°C) for frog thigh muscle.
FIG. 5. Relation of log decrement to duration of experiment at constant temperature (+19°C) for frog straight abdominal muscle.

muscle strips (longitudinal and transverse) were also used. The experiments were difficult because stomach muscle shows high "flow", being highly plastic, but Q showed a type of variation different from that for all the other muscles used. Curve B of Fig. 6 shows that Q falls by 18 per cent on going from +2°C to +12°C, thereafter rising, most sharply in the +12 to +25°C range.

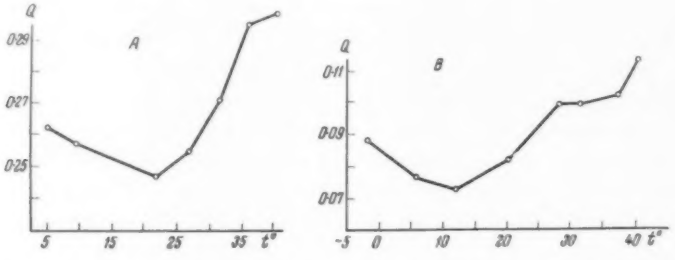

FIG. 6. Relation to log decrement to temperature for mollusc and frog smooth muscles. A: *Anadonta* adductor muscle. B: frog stomach-wall muscle.

Comparison of the curves indicates that, while showing the same general trend, the curves for the different muscles differ in detail, these differences being clear if the extensor and straight frog muscles are compared with the frog stomach and *Anadonta* adductor ones. In the first case dQ/dt is larger than in the second; although the results were not highly reproducible the general trend is clear. An additional check was effected using muscles in physiological saline instead of in a humidified chamber. The apparatus design was such that the torsional oscillations could be carried out in Ringer cooled to 0°C at the start and then gradually heated, tests being

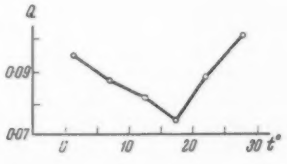

FIG. 7. Relation of log decrement to temperature for straight abdominal muscle on heating in physiological saline (initial temperature +1°C)

done every 30–40 min with the temperature steady, an ultrathermostat being used. The results agreed well with those from the previous experiments; Fig. 7, for straight frog muscle (Q vs. temperature) illustrates this.

DISCUSSION

The results for the various muscle types show that the general behaviour of the viscosity on heating previously cooled muscle is the same, although the curves differ in detail, and also vary even for a given type of muscle. This is particularly so as regards the minimum position; thus curves 1 and 2 of Fig. 2 show that for frog extensor muscle the minimum occurs in one case at $+16°C$, in the other at $+8°C$, the minimum on curve 1 being poor compared with that of 2; since the points at $+8°C$ and $+13°C$ are very close together it is difficult to be sure that the minimum does lie at $+8°C$.

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The curve shapes are obviously influenced to some extent by the time between measurements, i.e. by the number of points taken. Although the curve for any one muscle species is not strictly constant even if the initial temperature is fixed (e.g. 0°C), the kink in the extensor muscle curve is usually at a lower temperature than for the straight muscle or *Anadonta* adductor. Hence the secondary rise in viscosity must occur at higher temperatures (above $+20°C$) in the latter cases, the minima being thereby displaced to the right. The skeletal muscles show the sharper changes, whereas the others are smoother, dQ/dt being higher for the skeletal muscles.

In discussing the above material one should consider the muscle as a submicroscopic three-dimensional molecular network of long flexible protein chains which cannot slide freely over one another; all mechanical changes should be related to the state of the molecules in this network. The chains are of limited mobility, thermal vibrations being severely hindered by chain cross-linking. The resistance to deformation is primarily due to the high strengths of the longitudinal chain bonds, relative chain displacement without network disruption being the most probable process. Frictional forces (structural viscosity) thereby come into play and retard the relative displacements.

The high Q found at low temperatures is due to changes in the chains unrelated to structural damage, since the muscle remains transparent and excitable on cooling, or loses its excitability reversibly if more highly cooled. The high viscosity at about 0°C must therefore result from structural processes of a different type (as X-ray data also indicate [11]) from those occurring on strong heating, where the rise in viscosity is accompanied by a reduction in transparency and double refraction. Heilbrun [4] considers that the increase in viscosity in cooled living protoplasm is related to reversible coagulation in the proteins reminiscent of coagulation by heat. This effect is supposed independent of freezing, since it always occurs above the freezing point of water.

The structural changes occurring in living cells at low temperatures are at present unclear; the protein chains may aggregate, as has been demonstrated for protein solutions [3]. We suppose it possible that the structure is retained as a whole on cooling, but that the elements become more or less motionless, as thermal oscillations are reduced. The readily-ruptured interchain bonds may then become more stable and bind the elements into some sort of framework. This accelerates the chain

aggregation and hinders displacements in the chains or side-chains, which increases the internal friction. Colloid processes (gelling) may also occur in the sarcoplasm; any rise in temperature increases the relative mobility of the molecules, weakens the inter- and intra-molecular bonds, and so reduces the viscosity. Similar changes are found in high polymers, where the viscosity falls exponentially with rise in temperature [1, 2].

However, muscle tissue shows a secondary rise in viscosity at a certain stage, which indicates that the structural processes change their direction as the temperature increase continues. Comparison of our data with the reported data for various muscles and living objects indicates that this two-stage process is typical of protein systems. Nasonov and Aleksandrov [3] consider that the secondary rise always occurs within the sublethal range, and that it is always related to profound chemical changes, accompanied by simultaneous colloid changes. The chemical changes appear as aggregation and gelling. The molecular processes which cause structural damage to protein molecules here need special emphasis, because in most muscles the secondary rise occurs below the coagulation temperature, where excitability is entirely lost; we may suppose that here we get some pre-denaturation changes, primarily in the activities of the muscle proteins. It is now known that denaturation involves certain specific changes, such as the formation of new SH-bonds, hydrolysis and destruction of other SH-bonds, or molecular aggregation. Hydrolysis mostly results in irreversible denaturation, the intactness of the protein molecule being destroyed and denaturation completed. Other chemical reactions clearly occur before the protein structure is destroyed; these alter the molecular properties, and may play an important part in the actin-myosin interaction, leading to reduction in the order in the structure and increased internal friction.

It is thus clear that the viscous properties of protein systems (including muscle) follow a law different from that for polymers. The cause must mainly be the specific physiochemical properties of proteins, i.e. their tendency to coagulate at a definite temperature.

SUMMARY

The temperature variations in the mechanical properties of various types of muscle have been studied by torsional deformation. Two stages of change in the log decrement (viscosity) on heating muscle previously cooled to 0°C have been clearly demonstrated; the temperature variation curve as a rule shows a minimum. This is typical of all the muscles used, and evidently also for all protein bodies, but the minimum does not occur at a strictly constant temperature. The various muscles give different shapes of curve; frog thigh and abdomen muscles (Figs. 2 and 4) are to be contrasted with smooth frog stomach and *Anadonta* adductor muscles (Fig. 6*A* and *B*). The minimum varies even for one type of muscle, which may be related to temperature gradient variations and to the number of experiments performed. The temperature variations in the viscous properties of muscles and high polymers show major differences related to the secondary rise in viscosity at a certain temperature in muscle. The observed secondary rise is supposed to be related to pre-denaturation and denaturation changes in the muscle proteins (in the light of the theory of denaturation, Nasonov and Aleksandrov).

Translated by J. E. S. BRADLEY

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THE INFLUENCE OF TEMPERATURE ON THE RESTING POTENTIAL OF FROG NERVE*

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One of the first investigations on the influence of temperature on the resting potential was carried out by Bernstein [1] who used frog skeletal muscle. He found that temperature produced very small changes in muscle resting potential directly proportional to absolute temperature within a physiological range. According to Bernstein these results were in agreement with his membrane theory of the origin of bioelectric potentials, as expressed by the following formula:

$$E = (RT/F) \ln (C_i/C_o)$$

where R is the gas constant, T absolute temperature, F Faraday's number, C the concentration of K, and subscripts i and o refer to the inside and outside compartments respectively.

In the same paper, Bernstein (as well as Verzar [2] and Bremer and Titeca [3,4]) studied the effect of T on the resting potential of the sciatic nerve. Small changes in E of 0·08 mV/degree were found. Bernstein and Bremer and Titeca found a maximum value of E in the range of T between 16° and 22°C. Further cooling or warming of the nerve produced a small decrease in E. In order to investigate the relation of E to T recent work has involved the use of intracellular microelectrodes. A number of authors [5–8] working with muscle fibres of different animals, has confirmed Bernstein's findings that a decrease in T causes a very minute decrease in E. The temperature coefficient (Q_{10}) for E lies between 1·033 and 1·05, according to this

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data. Hodgkin and Katz [9] investigated the effect of T on E in the giant squid axon. Although this latter work reported small changes in E with a change in T, there are significant differences from the previous work in the field. While it had been maintained that cooling caused a small decrease in E, Hodgkin and Katz found an increase in E with a drop in T from 20° to 3°C.

The facts of some degree of relation between E and T have been used as an argument in favour of a Donnan equilibrium explanation for bioelectric potentials, i.e. that these potentials are physico-chemical in nature [1, 10]. Part of this view is that metabolic processes play an indirect role in maintaining the resting potential. They serve to maintain the completeness of the semi-permeable membrane, the uneven distribution of ions between the cell and the surrounding fluid, etc.

In relation to this, the work of Hodgkin and Katz [9] should be mentioned, which does not suggest a solely physico-chemical nature of E. If E were merely physico-chemical in nature, a fall in T should cause only a small decrease in E, rather than a rise as reported by Hodgkin and Katz. In addition, the argument in favour of a physico-chemical nature of E (metabolic processes play an insignificant role) is not in agreement with other work showing a relation of bioelectric potentials to metabolic processes [11–13].

In studying the relationships of various functional properties of nerve to a change in T, we have concentrated our attention on several details of resting potential changes observed on cooling nerve. The problem appeared even more interesting since there have appeared in the literature contradictions in interpreting the effect of T of the surrounding medium on E.

METHODS

The experiments were carried out on sciatic nerve of *Rana temporaria*. Two types of experiment were used to study the effect of cooling on E. In the first experimental series we applied overall cooling to the whole nerve. Isolated frog nerve was placed across two small paraffin vessels resting on the bottom of a deep warm container. The nerve was crushed in the distal chamber, and then covered with a 0·5–1·0 M solution of KCl. In the proximal vessel, where the nerve was intact, Ringer solution was used. Control experiments showed that under such conditions the injury potential reaches a plateau in a short time, after which it remains unchanged for 2–3 hr, only slightly decreasing towards the end of the observation period.

The temperature of the nerve was altered by means of submerging the lower part of the large container into a bath of cold water. Since this container had a relatively large volume, its cooling was quite slow, and consequently we could observe changes in E as a function of a gradual decrease in T of the surrounding medium.

Some authors [14], studying the effect of cooling on the physiological properties of nerve, have observed that similar experimental conditions require particular caution to prevent drying of the nerve, and condensation of water vapour on the preparation. This latter effect is due to the fact that, during cooling, the nerve remains somewhat warmer than the surrounding air, so that evaporation of water from it takes place. On the other hand, when the container is being warmed, the nerve shows a lag effect in that it shows a slower temperature rise than the surrounding air, so that droplets of distilled water condense on it. The portions of the nerve not immersed in the paraffin vessel were fixed with semi-solid petroleum jelly. The temperature of the

container was measured with an ordinary Hg thermometer, the bulb of which was placed in the same locus as the nerve. Temperature readings were carried out with an accuracy of 0·1°C.

In the second experimental series we applied local cooling to the nerve. Part of the nerve (usually the proximal end) was placed on a glass tube of diameter 11·5 mm, through which was circulated water of varying T. One of the measuring electrodes was placed on this section of the nerve. The indifferent electrode was placed on the adjoining section of nerve, which was either crushed or left intact.

Care was taken to prevent condensation of water on the nerve during cooling. To prevent such condensation two methods were used. In some experiments the glass tube passed through a small paraffin vessel with a few drops of Ringer solution. The vessel containing the nerve and the non-polarizable electrode was covered with a cover-glass. In other experiments the part of the nerve to be cooled was covered with semi-solid petroleum jelly together with the non-polarizable electrode. In both cases results were identical.

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The non-polarizable electrodes used were of the Dubois–Reymond type (Zn–$ZnSO_4$–Ringer), the wicks of which were submerged in the paraffin vessels, or placed directly on the nerve. The distance between the electrodes was 2–2·5 cm. The potential E was registered by a compensation method with a potentiometer of the PPTB-1 type. A mirror galvanometer was used as a null indicator (sensitivity $3{\cdot}5 \times 10^{-9}$ A) with which it was possible to measure a change of 0·05 mV accurately.

The dissected nerve was placed in Ringer for 30–60 min. The nerve was then, as described above, placed in a container for experiments with overall cooling, or on a glass tube in the series with local cooling. The potential difference between the two electrodes was measured over a period of time. Having ascertained that the baseline was constant, the nerve was cooled. Resting potential measurement was usually carried out 1 min after the onset of cooling. The duration of cooling varied from 15 to 100 min in various experiments. In order to bring about recovery from cooling, in the experiments with overall cooling, the entire chamber was removed from the cold water bath, while in the local cooling series, water at room temperature was passed through the glass tube.

RESULTS

In the first experimental series with overall cooling we observed that a gradual decrease of T in the chamber brought about biphasic changes in E. At the beginning of cooling, when there was a small decrease in T in the chamber, E gradually increased by 0·1–1·0 mV. With a decrease of 8–11°C below room temperature the second phase began with a small decrease in E (1·0–1·5 mV). When the water chamber was taken out of the container, and T came into equilibrium with room temperature, E showed a tendency to recover initial values. This recovery, however, was never complete, and sometimes it did not occur. Apparently this can be explained by the fact that E decreases absolutely over the course of the experiment, as seen in control experiments.

In this series 19 experiments were carried out. In 17 of them we observed a biphasic change in E on cooling. In one experiment, E remained unchanged during slight cooling, while later on it began to decrease. The last experiment showed a decrease in E from the beginning of the experiment. Fig. 1 illustrates one experiment

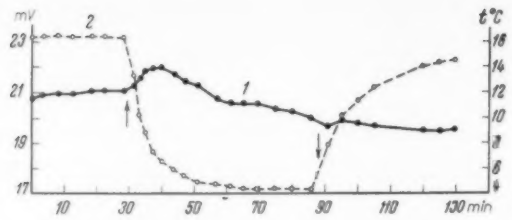

FIG. 1. Overall cooling of an isolated nerve. Room temperature 16·5°C. Prior to the experiment the nerve was kept for 1 hr in Ringer solution. *1* = resting potential in mV; *2* = *T* in degrees. Arrows show the beginning and end of cooling. Ordinate, resting potential; abscissa, duration of cooling. (Exp. on 26 September 1956, male.)

of this series. This figure, as with the others here presented, is a graphic presentation of protocol data. Every point on the curve corresponds to one measurement of *E*. The graph also shows changes in temperature of the chamber during the course of the experiment. Cooling first increases, then decreases, *E*.

In the local cooling series we observed analogous biphasic changes in *E* under the influence of cooling. The only difference was that with local cooling the two phases could be separated out. When the nerve was slightly cooled, i.e. water of 6–11°C below room temperature perfused through the tube, the reaction was limited to the first phase. The fall in *T* caused a rise in *E*, i.e. the cooled nerve section became positive to the other sections of the nerve. On the other hand, when the nerve was cooled by 12–18·5°C, *E* decreased without the first phase. The cooled segment became negative to the remaining nerve. As in the first experimental series, these changes in *E* were slight. With slight cooling, *E* increased by 0·1–0·7 mV, on greater cooling it decreased by 0·1–1·0 mV. The potential changes were reversible as a rule.

Altogether 32 experiments were carried out in this series, in which the effect of various temperature changes were investigated on the same nerve segment. In 16 experiments a slight decrease in *T* caused a decrease in *E* twice, no change twice, and a rise in the remaining 12. In 3 cases the initial increase was followed by a decrease. Such a biphasic reaction was found in those experiments where the decrease in *T* was in the 8–11°C range below room temperature.

Only once did a marked fall in *T* cause an increase in *E*, with a subsequent decrease, and in 2 cases no change was observed. In the remaining 15 experiments a marked decrease in *T* caused a fall in *E*. Results of two typical experiments of this series are shown in Fig. 2.

All the described experiments were carried out on fresh material, but in several cases the frogs used were kept for 2½ months previously under laboratory conditions. Out of 5 such experiments, a slight cooling only once caused a rise in *E*, once there was no change and in the remaining 3 there was a decrease. These findings permitted the assumption that the nature of the change in *E* depends to some degree on the functional state of the nerve. In order to confirm this assumption we carried out two additional experimental series.

First, the effect of local cooling was investigated on nerve *in situ* as well as on isolated nerve. The sciatic nerve was carefully freed up without injury to the circulation. A glass tube was placed under the freed nerve, through which water was

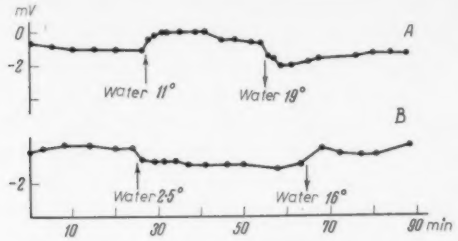

FIG. 2. Local cooling of an isolated nerve. *A* = slight cooling by 9°C. Room temperature 20°C. Nerve kept for 1 hr in Ringer solution before experiment. (Exp. on 30 November 1956, male.) *B* = marked cooling by 14·5°C, room temperature 17°C. Nerve in Ringer solution 45 min before experiment. (Exp. on 28 September 1956, male.)

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perfused at different temperatures. Tubes of 7 mm diameter were used in this experiment. Special experiments showed that this change in tube diameter had no effect on the results obtained. As in the previous experiments, the section of the nerve and the wick of the non-polarizable electrode were covered with semi-solid petroleum jelly. The indifferent electrode was placed on spinal or calf muscle. The baseline potential was usually rather large (10–20 mV on the average). At the beginning of the experiment it usually changed rapidly (more often decreased), reaching a plateau within 1½–2 hr. When *E* was constant, the nerve was cooled.

The results showed that the character of the changes in *E* on cooling was essentially the same with an intact circulation as with isolated nerve. Slight cooling caused a rise in E, marked cooling a decrease. The magnitude of these changes was considerably greater than with isolated nerve. While *E* of an isolated nerve changed by, at most, 1·0–1·5 mV in either direction, with nerve *in situ* changes in *E* reached +5·5 mV on slight cooling, and −6·5 mV on marked cooling. Examples of such experiments are shown in Fig. 3.

In control experiments, where circulation was intentionally interrupted, as well as in experiments where circulation was unintentionally occluded, changes in *E* on

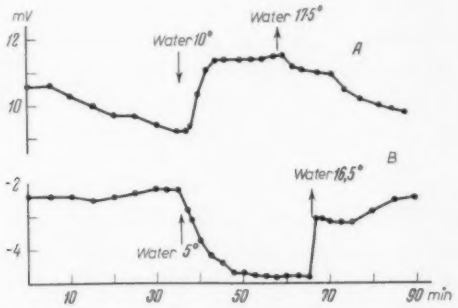

FIG. 3. Local cooling of a nerve with blood supply intact. *A* = moderate cooling by 9·5°C, room temperature 19·5°C. Indifferent electrode on calf muscle. (Exp. on 9 January 1957, female.) *B* = marked cooling by 13·5°C, room temperature 18·5°C. Indifferent electrode on spinal muscles. (Exp. of 7 January 1957, female.)

cooling were of the same order as experiments on isolated nerve. Consequently the decisive precondition for the large potential change appears to be an intact blood supply.

The last experimental series was carried out on nerves with a changed functional state, i.e. the nerve was treated with a 0·011 M solution of sodium iodoacetate, which selectively inhibits glycolysis. Control experiments showed that iodoacetate produced only slow biphasic changes in E: E increased very slowly at the beginning, then gradually returned to initial values, and finally began to decrease. In view of the slight change in E during the first phase, it can be stated that within 50–70 min after the addition of iodoacetate E remained practically constant.

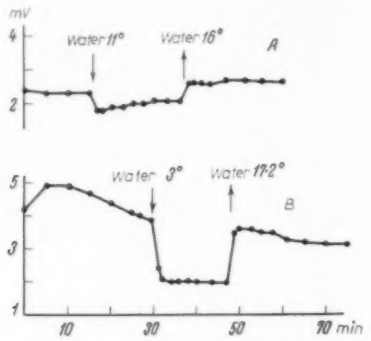

Fig. 4. Local cooling of isolated nerve treated with iodoacetate. A = slight cooling of the nerve by 7°C, room temperature 18°C. Nerve kept in iodoacetate solution for 20 min. (Exp. of 25 February 1957, male.) B = marked cooling by 16°C, room temperature 19°C. Nerve kept for 25 min in iodoacetate. (Exp. of 17 February 1957, male.)

The nerve was immersed in an iodoacetate solution for 20–30 min, then washed in Ringer solution and, as a rule, placed in a glass tube. Cooling was begun not later than 50–70 min after application of iodoacetate. In other words, cooling was applied to a nerve with an unchanged value of E, but with abnormal metabolic conditions. In this experimental series the first phase of potential change was never seen. Cooling led only to a decrease in E (Fig. 4). It should be added at this juncture that marked cooling of a nerve treated with iodoacetate caused 2–3 times as great a depolarization as in experiments with intact nerve. These potential changes, as well as changes in normal nerve, were fully reversible.

DISCUSSION

In his work entitled "Excitation, Inhibition and Narcosis", Wedenskii [15] pointed out that changes in resting potential, taking place under parabiotic conditions, can have a biphasic character. Electropositivity (prodromal flux) preceded in individual experiments a period of electronegativity in parabiosis. Proceeding from the findings of Wedenskii and the co-workers [16, 17] of Ukhtomskii, the latter [18] has proposed a theory to explain the biphasic character of changes of functional state in the course of a parabiotic process. According to this theory, the first phase of

parabiosis is characterized by an increase in lability, shortening of chronaxie and absolute refractory period, electropositivity, decrease in conductivity and by a number of other changes of functional properties of excited tissues. The second phase of parabiosis involves opposite changes in functional state: decrease in lability, lengthening of chronaxie and absolute refractory period, electronegativity, increase in conductivity.

As can be seen from the results presented in this communication, the resting potential also exhibits biphasic changes on cooling. Slight cooling increases E, further cooling decreases it. It has been shown above that the effect of T on E has been utilized to argue for a physico-chemical nature of the bioelectric potential. In our opinion, our results do not support such a view. The biphasic character of changes in E can be taken as evidence that nerve responds actively to a fall in T, rather than passively. This conclusion can be drawn from the experiments with isolated nerve, where the absolute potential change is slight. It holds even more for experiments carried out with nerves *in situ*, where the potential changes are greater. Further confirmation of the fact that the nerve responds actively to cooling lies in the character of the potential change as a function of the functional state of the nerve, and of metabolic processes. With an intact blood supply, i.e. with normal functioning of metabolic processes, this active response was even more marked. On the other hand, iodoacetate led to a disappearance of the first phase of the potential changes, and to a more pronounced second phase. It can be assumed that these changes in nerve response after iodoacetate are mainly the result of inhibition of metabolic processes, rather than due to a physico-chemical change (changes in membrane permeability, ion distribution, etc.), so that at the moment of cooling the initial resting potential of the nerve remained practically constant.

The results of our experiments help us to evaluate more critically the temperature coefficient. It has been shown several times in the biological literature that this coefficient cannot be used to analyse metabolic processes.* Without resorting to a detailed discussion of this problem, it can be stated that a calculation of this temperature coefficient has no point in the presence of biphasic changes; the same perhaps holds for other characteristics of the functional state of the nerve fibre. Were this not true, the temperature coefficient would change not only in absolute value, but this change would occur with a very small change in T.

The observed regularity of changes in E on cooling reveals the cause of the discrepancy in results reported in the introduction. The majority of workers found a potential decrease on cooling nerve or muscle. On the basis of our findings we see that these observations were actually observations of the second phase. Hodgkin and Katz described a rise in E on cooling in the giant squid axon, and this would appear to be only the first phase of potential change. A careful analysis of experimental data presented by the latter authors reveals that at lower temperatures (about 3°C) a slight decrease in membrane potential occurred, which was small enough (0·05 mV) to escape the attention of the authors. At the same time it is likely that this slight decrease indicates a transition of potential changes to the second phase. The biphasic character of changes in E on cooling can be used to explain the facts described by Bernstein and Bremer and Titeca, who found maximum values of E at 16–22°C. We can hardly agree with the explanation of the latter two authors that

* This problem is discussed in detail in the review article by Yel'tsina [19].

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a rise in T above this range injures frog nerve. The assumption of such damage by a rise in T is ruled out by those experiments of Bremer and Titeca where the effect of T on other functional properties of nerve was investigated, particularly on conduction rate and refractory period. Moreover, in one of Bernstein's experiments, a gradual cooling of the nerve increased E several-fold, and then decreased it.

As a rule, workers have not paid particular attention to the degree and rate of cooling. However, in several cases it follows from the published data that the cooling was marked [2, 6] and the potential measurements were carried out after some delay. This is a partial explanation of the fact that most authors have dealt with only the second phase of potential changes. Of course it cannot be stated that cooling of tissue is the only determinant of the direction and magnitude of the potential changes. In explaining the contradictory results from various sources, we must also take into account the variety of experimental material.

Our data can be compared with the results of Nasonov and Suzdal'skaia [14, 20, 21] who studied the effect of T on nerve excitability in several species. These authors conclude that nerves from poikilothermic animals possess a certain regulatory mechanism governing excitability with a physiologically adequate stimulation period (0·1 msec), and independent of T. It may be assumed that the first phase of resting potential change represents an electrophysiological adaptation of the nerve to change in T. It would be interesting to examine this assumption experimentally.

SUMMARY

(1) In experiments carried out on isolated frog nerve, cooled both locally and overall, biphasic changes in resting potential were seen. Slight cooling increased this potential, marked cooling decreased it. The absolute magnitude of these potential changes is very slight, not exceeding 1·5 mV.

(2) Local cooling with intact blood supply (*in situ*) caused similar biphasic potential changes, reaching higher values (+5·5 mV on slight cooling; −6·5 mV with greater cooling).

(3) Application of 0·011 M sodium iodoacetate caused a disappearance of the first phase of potential change, with a more marked second phase.

(4) The biphasic character of resting potential changes, as well as the dependence of these changes on the functional state of the nerve, and, above all, on metabolic processes, are taken as evidence that the nerve responds actively to a decrease of external temperature.

(5) The data obtained are not in agreement with a purely physico-chemical interpretation of these bioelectric changes.

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ELECTRIC POLARIZATION IN THE YOLKS OF HENS' EGGS*

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THE biochemical processes occurring in hens' eggs during development have been intensively studied, but the chemistry and biology of development is as yet far from being completely worked out, many contradictions still remaining. Less work has been done on the physical aspects; data are available on the pH, refractive index, electrical conductivity and viscosity of the albumin and yolk [1–4], but most of the studies have been done at intervals of 2–3 days, instead of daily, which greatly reduces their value. In our work the polarization properties of the yolk were studied, because this part of the egg is essential and has a definite morphological structure showing an electrical polarization which varies during development. To determine the degree of polarization we used conductivity measurements at two different frequencies [5, 6], the degree of polarization being given by the ratio of the low- and high-frequency resistances, R_l being the low-frequency resistance and R_h that at 1 Mc/s. At high frequencies polarization effects do not alter the conductivity, which depends solely on the free ion concentration, $R_l/R_h = k$, the polarization coefficient, being independent of the electrode area and spacing within reasonable limits. The frequencies used were 100 kc/s and 1 Mc/s.

METHODS

A special Wheatstone-type bridge system was used, two reactance bridges, for 100 kc/s and 1 Mc/s, being built into one unit. Sockets for the generator inputs and for the null detector, plus terminals for the unknown and standard impedances, were fitted to the front panel, a noninductive low-capacitance MSRB resistance box in

* Biofizika **3:** 2, 161–166, 1958 [Reprint Order No. BIO 123].

parallel with a variable 1000 pF capacitor being used as balancing impedance. The null detector was a shunted M 25–7 moving-coil reflecting galvanometer (sensitivity $0{\cdot}27 \times 10^{-9}$ A/mm at 1 m), a DGTs-7 germanium diode rectifier being fitted. The unit could be switched over from low- to high-frequency operation, or vice versa, so alternate measurements at both frequencies could be made without disturbance. The pure sinusoidal wave-shapes were checked on an oscilloscope; standard resistors were determined in order to evaluate the corrections required. The electrodes were platinum wires fused into glass tubes fitted with mercury contacts, and were fixed at a defined distance apart. "Russian white" hens' eggs were used, obtained from the Tomilin hatchery; they were incubated at 37·5°C. All the eggs were weighed before use.

In all, four series were run on the various components of the eggs.

Series 1. April–May 1956; k was determined for all components on each day of incubation.

Series 2. August 1956; as series 1, but unfertilized eggs were used as well, two eggs per day.

Series 3. December 1956. Only the yolk was studied, daily.

Series 4. February 1957. The yolk was studied, daily, measurements being made on the intact yolk and on specimens.

Four eggs developing normally were usually used (sometimes three); before opening they were weighed to check for evaporation loss. The various components were extracted with special pipettes; yolk and white specimens 0·5–1·0 cm^3 in volume were transferred to identical vessels placed in a water bath at 37·5°C. The measurements were made 15–20 min after transfer to the bath. In series 1 and 2 the liquid fraction ("new plasma") was sampled as well as the white and yolk, i.e. the amniotic and allantoic fluids. In series 4 intact yolks were used; here the method used varied according to the stage of development. In the early stages, measurements were made at various depths under the developing embryo; as from day four the "new plasma" was removed, after which the top and some lower layers of the yolk were examined. At the end of the incubation the embryo plus yolk sac was removed from the egg, the yolk sac opened and measurements made directly in the yolk. In all cases k was calculated from the results.

RESULTS AND DISCUSSION

The "new plasma", white, allantoic and amniotic fluids gave $k \approx 1$ at all times, i.e. they showed no polarization at 100 kc/s (Fig. 2). The yolk, however, gave $k > 1$, the value varying considerably during incubation, the absolute magnitudes of the changes differing between series, but the mean values for all series showed the same general trends (Fig. 1); the pre-incubation k was usually 1·15–1·2, rising to 2 or more in the first few days. In all cases marked rises in k were found on days 5–6, 8–9 and 11, a sharp drop occurring on day 14, falling to nearly one as from day 15, as the embryo began to take in the residual yolk plus sac. The larger rises in the second series are due to the experiments being done in August, i.e. on eggs laid in the summer, whereas all others were done on eggs laid while the hens were on winter feed; biologically speaking, the eggs were not equivalent, as several workers have observed that the chemical compositions and physico-chemical properties of white and yolk are affected by the feed [7, 8].

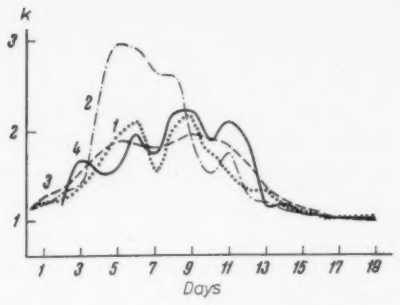

FIG. 1

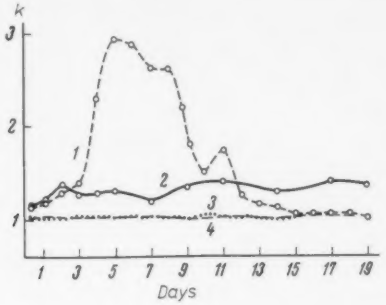

FIG. 2

FIG. 1. Changes in polarization factor in developing egg yolks from four series of experiments. *1–4* are the respective series.

FIG. 2. Changes in polarization factor in the white and yolk of incubating fertile and infertile eggs (second series of experiments). (*1*) Yolks of developing eggs (*2*) yolks of unfertilized eggs, (*3*) whites of developing eggs, (*4*) whites of unfertilized eggs.

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The unfertilized egg yolks (Series 2) showed very different effects from the fertile ones; if new-laid, k was about the same for both, rising for the unfertilized in the first few days and then remaining between 1·2 and 1·4 throughout (Fig. 2). To establish how well the *in vitro* measurements reflected the true properties we made measurements directly on the yolk, doing as little damage as possible, thereafter immediately taking normal samples; the values of k in the intact yolks were found to be higher, the difference being considerable, though the general trend was maintained (Fig. 3), except in the first few days. This latter aspect will be discussed below.

It is thus clear that the egg yolks showed marked polarization effects at low frequencies; one reason for the regular changes found in the yolk may be the structures which develop, but which are not constant. The yolk undergoes complex changes, not purely destructive but, rather, reconstructional in nature; hence the difference

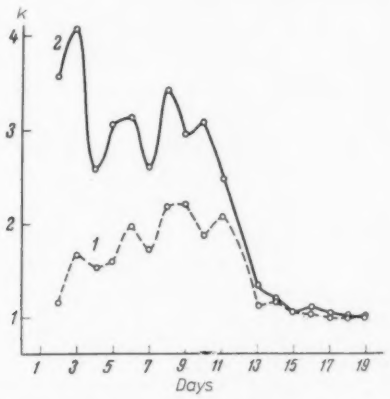

FIG. 3. Changes in polarization factor in developing egg yolks measured *in vitro* (*1*), and in the intact yolk (*2*).

from the *in vitro* value may be due to the mechanical damage to the structure during sampling. This was checked by stirring the yolk in the tubes vigorously with a glass rod, followed by repeat measurements; this altered k markedly (see Table 1). The chemical transformation of the yolk into the cells of the chick is effected by a large variety of enzymes; the fresh-laid egg before incubation contains much less of these than does the developing egg, in which the enzyme content steadily increases; some data on the activities of various enzymes have been published. However, such data are not numerous and are often contradictory, e.g. those on yolk catalase, which has a part in respiration; Sokovnina [9] states that the activity rises during days 3–6, then remains constant to day 9, followed by a drop which reaches the normal level by day 15.

TABLE 1. EFFECT ON k OF STIRRING YOLK

Days of growth	k	
	Before stirring	After stirring
9	2·285	1·164
9	2·230	1·040
9	2·080	1·120
9	2·080	1·190
11	2·220	1·315
11	2·458	1·334
11	2·170	1·206
13	1·180	1·022
13	1·160	1·008

On the other hand Davydov [10] states that the activity is maximal on day 15; Bruslavets's data [11] on the oxygen uptake are here of interest. Two maxima on the uptake curve (at days 5 and 10) are found, plus a slight rise at day 7. These rises in oxygen uptake regularly precede the rises in k found in our experiments by one day, while our findings of a rise on day 2, with a maximum at 8–9 days and a fall thereafter to day 15 agree with Sokovnina's data. The changes in k thus seem to correspond to the changes in the rates of oxidative processes in the yolk. Bruslavets also states that seasonal variations in oxygen uptake occur, the maximum being found in eggs laid in the summer, which corresponds to the high k values found in our second (August) series.

Our measurements on intact yolks showed a particularly high k during the first three days, the values found much exceeding those for extracted specimens; this is because the measurements were here made directly under the embryo, whereas the yolk specimens were taken from remote yolk areas; the metabolic activity immediately below the embryo might well be expected to be much higher. However, the yolk was of non-uniform activity throughout the incubation, as layer-by-layer measurements showed (Fig. 4). The data indicate a polarizability gradient away from the embryo.

At present the above data cannot be satisfactorily interpreted, though it is clear that particles with dipole moments must be present; these particles may be unstable and be destroyed by mechanical removal. The developing egg is a labile biological

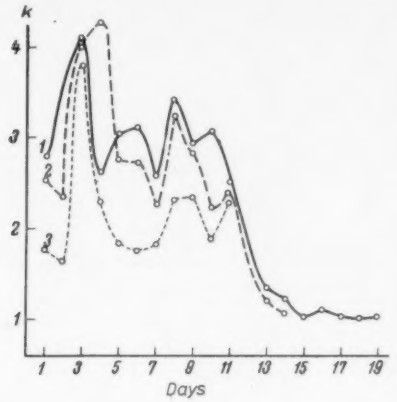

FIG. 4. Changes in polarization factor in different layers of developing egg yolks. (*1*) Surface layer 0·5 cm thick, (*2*) at 0·5–1·0 cm, (*3*) 1·0–1·5 cm layer.

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system, in which the complex biochemical processes involve an extensive molecular reconstruction, and hence changes in the electrical properties. Further investigations may throw more light on these processes and their role in development.

SUMMARY

(1) The yolks of hens' eggs show polarization effects in low-frequency fields.

(2) The various layers of a fresh egg yolk before incubation show different polarizabilities, the highest occurring in the white area directly beneath the embryo disk and in the latebra.

(3) These nonuniformities persist during the first half of the incubation.

(4) The polarizability of the main yolk varies considerably during incubation, three marked rises being found at about days 5–6, 8–9 and 11; the polarizability vanishes as from day 15.

(5) Unfertilized eggs show scarcely any change during incubation.

(6) *In vitro* measurements give polarizability indices much lower than are found for minimally damaged yolk.

(7) The polarizability is almost completely destroyed by stirring the yolk specimen vigorously with a glass rod for a short while.

(8) The changes in yolk polarizability correlate with the changes in the rates of oxidative processes and are evidently due to structural changes in the yolk.

Translated by J. E. S. BRADLEY

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LIGHT-INDUCED CHANGES IN THE ION BALANCE IN ALGAL CELLS*

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THE effect of light on the distribution of materials between plant cells and their surrounding medium has long been studied; it has been established beyond doubt that light intensifies the absorption of mineral constituents into plant cells. It is claimed [1, 2] that the light increases the permeability of the cell membrane; Hoagland and Davis [3] consider this an inadequate explanation and suppose that the light energy enables the cell to transport material up a concentration gradient; Ingold adopts the same view [4]. Scott and Hayward [5] have obtained interesting data; the effects of light and monoiodoacetic acid on the ion balance of sea algae were studied; these cells show higher K and lower Na than the sea-water. The cells were found to lose some K to the sea-water in darkness and to take up some Na, i.e. the ions were redistributed, travelling down the concentration gradients. The reverse occurred on illumination. The monoiodoacetic acid tests showed that the dark effect was accentuated, but no effect was found if the cells were illuminated in flowing sea-water. It was supposed that break-up of intermediate photosynthetic products and glycolysis provided the energy to transport ions up the gradient. The mineral constituents of the cell were supposed predominately in the free state.

Latterly much more information has accumulated; it indicates that most of the mineral is chemically combined with or bound to the colloids; we may then suppose the redistribution occurring on illumination to result from a change of concentration gradient. In the present work we studied the redistribution process, while allowing for the intracellular concentration; ion balance changes were studied by the electrical conductivity of algal cells.

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METHODS

The resistance of a system consisting of filamentary algae plus nutritive medium was studied, also the resistance of a suspension of single algal cells, as affected by illumination, high-frequency techniques being used. Living tissues show a drop in resistance with rise in frequency, this being due to electrical polarization effects in the cells. Above 10^6 c/s these polarization effects do not influence the resistance, which is determined by the free electrolytes in the cell.

A Wheatstone bridge was used with the filamentary algae, two symmetrically disposed arms being comprised of non-inductive resistors of low capacitance and known ratio. The unknown resistance was found by balancing with a resistance box; since the impedance has a capacitative component, a 500 pF variable capacitor was connected in parallel with the variable resistance. The 10^6 c/s source was a valve generator, the null detector being a mirror galvomometer of sensitivity $0{\cdot}32\times10^{-9}$ A/mm at 1 m, a germanium diode rectifier being used; the accuracy was 0·1–0·2 per cent. A plastic cuvette of area 20·5 cm^2, fitted with brass electrodes each of area 6·5 cm^2, was used.

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The sample consisted of 350 mg of algae plus 10 ml of solution, all samples for any given experiment being taken from the same stock flask; the samples were kept in a thermostatic enclosure, the temperature being kept constant to 0·1°C. The alga chosen was *Cladophora* (green), cultivated in Knopp's solution diluted by a factor of four with distilled water; air was periodically blown through the culture, and if the daylight was inadequate artificial lighting of daylight type was used.

A similar arrangement was used with the single-cell cultures, though the null detector was an oscilloscope, the accuracy being 0·5 per cent. In these experiments the resistance of a cell could be calculated from Maxwell's equations, which have also been used for a similar purpose with erythrocytes. The equation is of the form

$$(r_1-r)/(r_1+2r) = \rho(r_1-r_2)/(r_1+2r_2)$$

where r is the specific resistance of the suspension, r_1 that of the medium and r_2 that of the particles, ρ being the volume concentration of the latter. A glass vessel was used with platinized silver needle electrodes inserted to a defined depth; the resistance was measured, then the suspension was centrifuged for 3 min at 5000 rev/min and the resistance remeasured. The vessel was calibrated by performing measurements on solution of known specific resistance; r_2 is thus easily found. We used *Chlorella*, grown on Craig's medium under natural lighting, plus aeration for 12 hr per day.

In all tests on lighting effects, filament lamps were used, these being fed from a fixed voltage during the tests. To avoid heating effects the samples were shielded with 8 cm water filters; in some cases a heat-absorbing SZS-14 glass filter (transmitting only a small fraction of the short-wave infra-red) was also inserted. The light intensity was measured with a thermopile and expressed in erg/cm^2 sec. Both types of test were done six times, though on occasion we only give the results from 3–4 sets here, because not all the data could be averaged on account of the conditions being different.

RESULTS AND DISCUSSION

The resistance of the filamentary suspension increased on illumination and fell in the dark. The dark resistance was measured by keeping the sample in the dark in

the thermostat for an hour before measuring the initial resistance and then putting on the light. Measurements were made every 20 min for 4 hr, in the light for the first 2 hr and in the dark for the last 2 hr. Table 1 gives the results. The rise in resistance undoubtedly results from the action of the visible light, because the infra-red would heat up the system and reduce the resistance. This effect was demonstrated by using a piece of IKS-1 coloured glass, which together with the water filter selects the 800–1300 mμ region.

TABLE 1. RESISTANCE CHANGES AT 21°C AND $2{\cdot}89 \times 10^2$ ERG/CM² SEC USING A WATER FILTER

Conditions	Time (min)	Resistance (Ω)	As % initial
Light		initial	
		7016	100·0
	20	7103	101·2
	40	7223	103·2
	60	7330	104·5
	80	7446	106·1
	100	7562	107·8
	120	7691	109·5
Darkness			
	140	7555	107·6
	160	7447	106·1
	180	7372	105·0
	200	7306	104·0
	220	7246	103·2
	240	7180	102·3

The changes depend on the previous illumination history; if the illumination prior to test was the same as during the test there was no real change, while the changes were large if the sample was kept in the dark before an experiment in the light, or vice versa. Fig. 1 shows the mean results from three tests in which the specimens were kept in the dark for various times before testing; it is clear that the light-induced rise depends on the length of the dark period. In all subsequent tests on light effects the samples were kept in the dark previously for 20 hr, and conversely; then, if the algae comprised not more than 3 per cent by volume, the change in resistance in the system was clearly mainly due to change in the solution. In some cases the changes in the solution resistance were measured as well; the two changes were in the same direction.

In this way the time variations were studied, sometimes for as long as 7 hr; the rate of change was found to fall in the later stages in both cases (Fig. 2). The relations of rate of change to light intensity and spectral composition are of considerable interest; the effects of light intensity change (other conditions being the same) were studied using physiologically active radiation at 1·08, 2·16 and $4{\cdot}33 \times 10^3$ erg/cm² sec. The results (Fig. 3) show that in this range the rate of change is almost exactly proportional to the intensity.

To study the effects of spectral composition we used blue and red light; red was eliminated with a SZS-18 glass filter, which selects the 400–550 mμ region, and blue

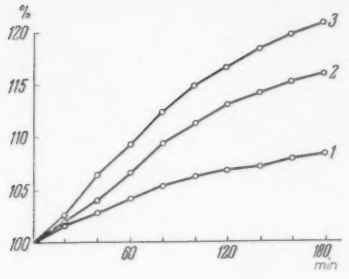

FIG. 1

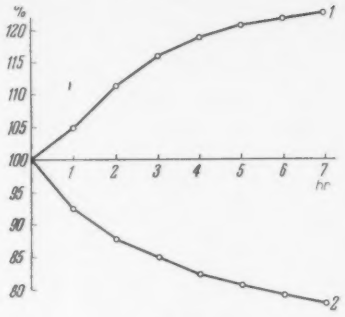

FIG. 2

FIG. 1. Light-induced resistance changes vs. previous dark period. Ordinate: resistance, % initial. Temperature 20·5°C, intensity $3{\cdot}61 \times 10^3$ erg/cm² sec, water filter. (*1*) specimen in dark 1 hr, (*2*) 5 hr, (*3*) 15 hr.

FIG. 2. Time variations of resistance in light and dark. Ordinate: resistance, % initial. Temperature 21°C, intensity $3{\cdot}61 \times 10^3$ erg/cm² sec, water filter plus SZS-14. (*1*) light, (*2*) dark.

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with a KS-10 plus a SZS-14, which combination selects the 600–750 mμ range. The two colours were made of equal intensity or of equal numbers of physiologically active quanta. In the first case the intensity was $2{\cdot}16 \times 10^3$ erg/cm² sec, while in the second the blue was of the same intensity but the red was of intensity $1{\cdot}44 \times 10^3$ erg/cm² sec.

Fig. 4 shows the results; even when the numbers of quanta were equal (Fig. 4*B*) the red light produced a greater effect, the blue producing an average change of 7·2 per cent in 3 hr, while the red gave 9·6 per cent. The difference is small but significant, since in all cases without exception the red was more effective; also the differences were several per cent at the end, whereas when samples were tested under identical conditions the differences did not exceed 0·5 per cent. The difference between the two may really be greater, because the red filter transmitted a certain amount of infra-red.

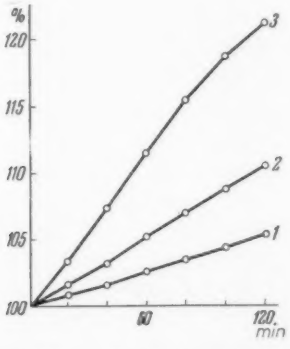

FIG. 3. Resistance variations at different light intensities. Ordinate: resistance, % initial. Temperature 21·5°C. Filters water plus SZS-14. Light intensities (1) $1{\cdot}08 \times 10^3$ erg/cm² sec, (2) $2{\cdot}16 \times 10^3$, (3) $4{\cdot}33 \times 10^3$.

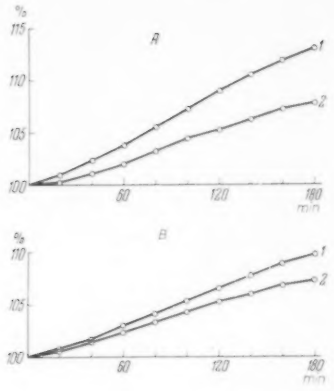

FIG. 4. Resistance variations at different light qualities. Ordinate: resistance, % initial. Temperature 20·5°C. (*A*) identical light intensities, (*B*) identical numbers of quanta. (1) red, (2) blue light.

It was therefore supposed that the changes are due to ion transfer between cells and medium, the rise in resistance of the solution indicating that the cells absorbed the ions, and conversely. It is known [6] that $Ca(HCO_3)_2$ solutions containing water plants show a drop in conductivity during the day, and a rise at night. In this case the weakly bound CO_2 is used up during the day and $CaCO_3$ precipitates, while at night some respiratory CO_2 causes the $CaCO_3$ to dissolve. Unfortunately we did not determine the rate of assimilation or allow for the form of the CO_2, but we may suppose that free CO_2 and HCO_3^- ions existed in a definite relationship in our solutions; if the free CO_2 was inadequate for photosynthesis the resistance changes are clearly due to the same causes as in [6]. The following test was done: one sample was kept in the light and another in the dark; the algae were then transferred to fresh solution and the respective resistance changes in light or darkness followed. It was found that algae placed in identical solutions but subjected to different illumination conditions showed different resistance changes; a rise of resistance on illumination and a drop in darkness were found. These effects can hardly be explained by the absorption of weakly bound CO_2 alone, accompanied by carbonate precipitation, etc.

A medium containing both carbonate and bicarbonate becomes more alkaline during assimilation [6–8], these changes being due to CO_2 consumption. In some of our tests the pH was measured, but it was found that 4 hr illumination produced, at most, a rise of 0·1–0·2 units; in darkness the converse was found. These slight changes are due to the solution being heavily buffered.

It was of interest to see if the resistance changes persisted when CO_2 was supplied freely, air being blown through the vessel for this purpose. Samples containing 900 mg of algae in 50 ml of solution were placed in test tubes fitted with glass tubes to admit air. Two were kept in the dark and two in the light for 20 hr, 10 ml samples being then taken and tested for pH and resistance at 20·5°C. In all, the air was passed for 28 hr. The rise in the light and fall in the dark were again found (Table 2).

Under these conditions the algae were continuously supplied with atmospheric CO_2, so the changes in the illuminated tubes must have been due to ion transfer.

TABLE 2. CHANGES IN RESISTANCE AND pH OF SOLUTION IN 8 HR (EXPERIMENTS USING AN AIR CURRENT)

Lighting conditions during the 8 hr	Test No.	Sample No.	Resistance (Ω)		pH	
			Initial	Final	Initial	Final
Light	I	1	6490	7001	7·0	7·0
		2	6511	6854	6·9	6·8
	II	1	6811	7118	6·8	6·8
		2	6754	7155	6·7	6·8
Darkness	I	3	7287	6622	6·7	7·0
		4	7101	6590	6·8	7·1
	II	3	7657	7031	6·6	6·8
		4	7788	7068	6·5	6·9

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The effects in the darkened tubes might have been due either to ion transfer or to CO_2 changes. The pH differences between tubes were very small, though the solutions became somewhat more alkaline in the dark. Ingold [4] has found this effect in tests on light-induced ion shifts in *Elodea*; he explains it as due to a relatively alkaline bicarbonate buffer mixture forming in the dark, and this explanation may be applicable here.

The following direct test of the causal connexion between conductivity change and ion balance was done. Algae were kept in the dark in tap-water and then placed in Knopp's solution, prepared with tap-water and then diluted three-fold with it. The usual comparison between "light" and "dark" samples was performed; the resistance rose on average by 8·3 per cent in 2 hr on illumination, but there was also a very slight *rise* in the "dark" sample (Fig. 5). Here the algae were in solutions of high ionic strength, and so the cells would be expected to absorb ions both in the light and in the dark; the larger change in the light is clearly due to more marked ion absorption.

In the last twenty years the view that ion absorption by plants involves mainly sorption has become entrenched in plant physiology. Sabinin [9] adopts this view; so do Brooks [10], Mazia [11] and Ratner [12]; the view also accords with the sorption theory of permeability. In this theory some part of the inorganic element is bound to the colloids and some is in solution in the protoplasm, the ion concentration being determined by the solubility in protoplasm. Ion balance changes then involve changes in colloid sorption [13], so light may perhaps cause changes in the sorption properties. The following result is of interest in this connexion; algae placed in distilled water produce a fall in the resistance, but the changes are slow and occur only during the first hour if the solution is illuminated, while they are more rapid and persist for 2 hr in the dark (Fig. 6). We may suppose that some ions desorb and leave the cell in the dark, whereas, in the illuminated cells the free ions try to diffuse out but are largely unable to do so. If this is so it must account for the fall in resistance; it would then indicate that the sorption properties of illuminated cells are more marked than those of unilluminated.

The resistance changes in the cells themselves are of some interest; these were studied on suspensions of *Chlorella*. Volume concentrations of 22–25 per cent were

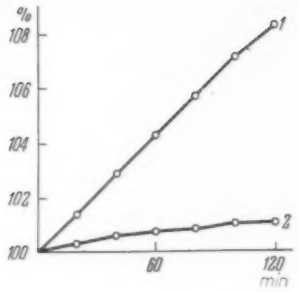

FIG. 5

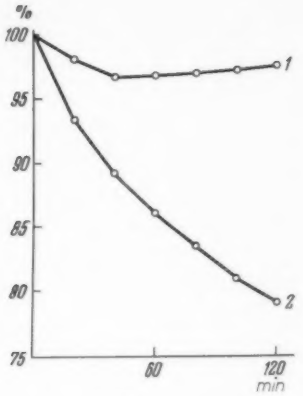

FIG. 6

FIG. 5. Time variations of resistance in light and dark using algae kept in tap-water and Knopp's solution. Ordinate: resistance, % initial. Temperature 24°C. Filters water plus SZS-14. Light intensities $2{\cdot}89\times10^3$ erg/cm² sec. (*1*) light, (*2*) dark.

FIG. 6. Time variations of resistance in light and dark using algae kept in a nutritive solution and distilled water. Ordinate: resistance, % initial. Temperature 24°C. Filters water plus SZS-14. Light intensities $2{\cdot}89\times10^3$ erg/cm² sec. (*1*) light, (*2*) dark.

used; the initial resistances of medium and suspension were measured and samples previously kept in darkness were illuminated at $3{\cdot}24\times10^3$ erg/cm² sec, water and SZS-14 filters being used, the tests lasting 3 hr, after which the final resistances of suspension and medium were measured. The cell resistance was found to be increased by illumination and to fall in the dark (Table 3). The rise in resistance in the light supports the idea that the ions are more extensively bound.

TABLE 3. SPECIFIC RESISTANCES OF SUSPENSIONS, CELLS AND MEDIA

Conditions	Test No.	Volume concentration (%)	Specific resistance (Ω)					
			Suspension		Medium		Cells	
			Initial	Final	Initial	Final	Initial	Final
Light	1	25	636·9	781·3	613·5	763·4	724·9	828·7
	2	22	666·7	800·0	645·1	787·4	740·8	862·7
	3	22	751·9	934·5	735·3	917·4	805·7	1005·2
	4	25	681·7	828·5	675·7	823·0	703·8	857·2
Darkness	1	25	813·0	653·6	800·0	636·9	868·5	720·0
	2	25	815·7	662·3	813·0	653·6	822·9	688·1
	3	22	888·9	719·4	885·0	704·2	905·2	771·6
	4	25	847·5	680·2	840·3	671·1	873·9	710·9

CONCLUSIONS

High-frequency resistance measurements give data on ion balance changes in algal cells; filamentary and single-celled algae were used in suspension with various illumination conditions. The resistances of suspension and solution increase on illumination and fall in the dark, because the cells absorb ions from the solution in the light and lose them in the dark. The ion redistribution in response to visible light has been studied in detail; the amounts entering the cells depend on the previous illumination history, the uptake in the light being the larger the longer the previous period in the dark; the effect is also dependent on the light intensity. Comparison of blue and red lights shows the latter to be more effective. The rate of transfer at different ionic strengths in the solution has been related to the illumination conditions.

The experiments with *Chlorella* are of particular importance in elucidating the light-induced ion uptake; the cell resistance increases on illumination and falls in the dark. Some of the intracellular ions may be supposed to become adsorbed or chemically bound when the light acts, so the reduced intracellular free ion concentration causes ions to diffuse in, the converse process occurring in the dark. If this is so, it is not the permeability but the concentration gradient that is affected by the light. The results indicate that there may be a relation between increased mineral uptake and photosynthesis.

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THE CAPACITY OF LIGHT-SENSITIVE SYSTEMS TO DIFFERENTIATE RADIATIONS*

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1. WE have previously expressed the view that to "see" some object always means to differentiate it from a precisely defined group of other objects [1]. So, if the objects from which our given one is to be differentiated are not defined the problem of whether it can be "seen" is indeterminate. We endeavoured to show, using several examples, that neglect of this in studies on vision has led to misunderstandings, apparent contradictions and erroneous conclusions.

While the examples given in the previous paper related to certain particular phenomena in human and animal vision, here we deal with a wider range of topics, beginning with a survey of a technical problem, where the receptor is not the eye of some living being but a multilayer colour emulsion, as used in colour cinephotography. Although such a film is artificial, it records radiations, and therefore the negative–positive colour process to some extent resembles a colour vision system. We shall not touch on the rather complex problems of the general theory of colour reproduction, but will restrict consideration to the particular problem of reproducing fine coloured detail.†

Such films consist of three layers, the uppermost being blue-sensitive, the middle green-sensitive and the bottommost red-sensitive (it would be more exact to say that they are sensitive to the short-, medium- and long-wave sections of the visible spectrum). After development the film shows three images: top layer yellow (absorbing blue), middle purple (absorbing green) and the bottom blue (absorbing red). Not only have the light areas become dark on the colour negative; the colours have been replaced by their complements. Light yellow appears as dark violet, light blue as dark brown, dark brown as blue, etc. On printing on to a colour film from the negative the colours are again reversed and a picture more or less corresponding to the object results.

Of the three layers in the film the bottom one is of lowest resolution and the top one of highest, because the light is somewhat scattered on passing through the layers. This must also result in details of different colours also being unequally sharp; it seems at first sight clear that blue objects will give sharper negative images because the blue rays act on the top layer, while red ones (which act on the bottom) must give less sharp images. The negative image colour is approximately complementary to that of the object, and therefore details which are sharpest on the negative should

* Biofizika **3**: 2, 178–183, 1958 [Reprint Order No. BIO 125].

† The description of the negative–positive colour process given here is extremely brief, as it is assumed that most readers have a general acquaintance with the principles of colour photography.

lose relative sharpness on printing, and conversely, the final conclusion being that details of any colour would be produced about equally sharply. This argument is actually used and is convincing to many, but is erroneous.

When the sharpness in reproducing some details is discussed we cannot restrict the discussion to the colour of that detail alone, but should state also the colour of the background on which it is seen. Suppose we photograph a blue line on a white background; the blue mostly reflects blue rays, to which the topmost layer is sensitive, while the white background causes all three layers to develop. The topmost layer will thus receive light from both, so there will be no image of the line in the top layer, but just general blackening. In the two lower layers the blue line gives no blackening, unlike the background; in these we thus get an image as a transparent line on a dark background, so the blue line will give a negative image as a yellow line on a dark background, of sharpness determined solely by the resolutions of the two lower layers (which form the image); the resolution of the top layer has no effect (provided the line is on a white background).

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Suppose now we have the same line on a black background; the negative will here only be blackened in the topmost layer in the position of the line, while the black background produces no effect, so the image is here in the top layer, but as a dense yellow line on a transparent background. Its clarity will be determined by the resolution of the top layer. We may thus say that the sharpness of any recorded colour detail is not determined by which of the layers reacts to its colour, but by the one which records the difference between detail and background, i.e. in which layer the image lies. This is precisely analogous to an insect "seeing" the colour of a poppy; the sensitivity of the eye to the colour reflected by the object is not the important feature; rather it is the power to differentiate the red poppy from green grass.

Details of a given colour have different sharpnesses on the negative, dependent on the background colour, while details of quite different colours may be identically sharp. Thus, a yellow line on a white background, a blue on a black, an orange-red on a purple, or a green on a blue, etc., all give images confined to the topmost layer. We can say that these colour pairs have "identical" colour differences, though which colours are in this sense "equally" different depends on what light-sensitive receptors are involved (here, what the spectral sensitivity curves of the three emulsion layers are).

It is clear that although the negative and positive colours are very different, the colour differences between detail and background are retained; a yellow line on a white background gives a blue line on a black on the negative, while on the positive we get a yellow on white again. Neglecting colour distortions so introduced, the images on negative and positive will lie in the same layers. Hence those details least sharp on the negative become still less so in the positive, and conversely; this clearly directly contradicts the conclusion above. We give an example where an incorrect approach gives an erroneous answer to a well-defined question.

The "identical colour difference" concept, which applies to very different pairs of colours, is useful in studying colour vision as well as in colour reproduction technology.

2. The "to see is to differentiate" principle is of value in deciding the general methodology of experiments, as well as in particular problems in physiological optics.

Smirnov and Bongard [2] have pointed out numerous advantages of their colorimetric method for determining the spectral sensitivities of receptors over the threshold method. In particular, they quite correctly observe that the latter gives in principle incomplete data if there are several receptors with differing spectral curves, i.e. in all cases of colour vision. We do not repeat their arguments, with which we agree completely, but merely point out that the advantages of their method are closely related to their using the approach discussed here; the present arguments are to a considerable extent the reason why colorimetric principles are used in electrophysiological vision studies on animals in our laboratory. The major difference between the two methods is as follows; in colorimetric tests radiations are compared in pairs, so a series of such tests provides an exhaustive answer to the problem of which radiations are differentiable from one another and which not, for that colour vision system. In other words, they give the most direct and complete indication of what the system can "see", since the power to "see" is always determined by the power to differentiate radiations from one another.

In threshold tests only "seeing" in darkness (differentiation from darkness) is tested; in essence they involve the unrealized assumption that "to see" means "to react to light". We have already pointed out that this approach is incorrect and gives incomplete data. The difficulties in determining the number of independent receptors by threshold methods* are remarked on by Smirnov and Bongard; they stem directly from this cause. While the purely practical advantages of colorimetric methods appear most clearly with colour vision (multiple receptor) systems, their advantages of principle are best illustrated for single-receptor systems (no colour vision). In this latter case it would appear that spectral sensitivity determination by threshold ought to give the same result as colorimetric methods for two identical radiations. But this is only so if it is shown by experiment that the additivity law is correct for the system; this has been done for man, and it is one of Grassman's laws. It was deduced from colorimetric tests.

In studying animal vision, proof of the additivity law must come first, since if it does not apply the functioning of the receptor cannot in principle be described completely by any spectral curve, however well defined. Hence a curve found by threshold is unsuitable for solving radiation differentiation problems if the stimuli are above threshold; calculations with such a curve for other than threshold stimuli will be incorrect. So, even if we find a curve in this way it is of no use if the additivity law has not been proved, and this latter can only be done from colorimetric tests; if it is, then not only do the latter give numerical values for the spectral curve, but they also prove that the curve can be used to determine whether any pair of radiations of given spectral compositions are differentiable.

Monochromatic radiations are mainly used in both methods, but the colorimetric enables one to solve differentiation problems for radiations of any spectral composition; while the threshold, strictly speaking, relates solely to monochromatic radiations. If some system where additivity is *not* obeyed should be found the system could not be specified at all from absolute threshold determinations, while the colorimetric, in which radiations are compared in pairs, gives a complete answer as

* Granit [3] quite incorrectly claims enormously more different types of receptors than actually exist in his animals.

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to what radiations are differentiable for any system, whatever its peculiarities, in particular deviations from additivity.

The "to see is to differentiate" theory thus necessarily implies that any unknown vision system must first be studied colorimetrically.

3. Iarbus [4–6] has obtained very valuable data on human colour vision from studies on images fixed and moving relative to the retina; some of his conclusions are of interest here.

When the retinal imaged is fixed and unchanging in time an "empty field" forms; within this no differences are seen and the image therefore vanishes, but the field retains a colour dependent on the conditions. In particular, if the field occupies only part of the retina and is projected on a uniform background it takes on the colour of the latter; the field is then, as it were, an artificial blind spot; a region of the image projected on this spot also takes on the colour of the surround.

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Analogies have been drawn between the empty field formation process and the pulse train transmitted along a nerve fibre on replacing one radiation by another; on this view the field forms when the pulses cease. In other words, the empty field corresponds to an area of the retina at which no information (signal) arrives; the similarities between an empty field and a blind spot (which receives no signals) are then quite clear. It is found that when any object (e.g. a shadow [6]) moves over the empty field its colour alters, but the difference between the apparent colours of object and field reproduces in a certain sense the difference between their actual colours. We naturally explain this as due to the background radiation being replaced by that from the object at their common boundary, the receptors recording this difference. In any case, it is quite clear it is not the colours of the fixed and moving images that are recorded, but only the nature and magnitude of the colour step.

It is sufficient to record such colour differences in order to see the object (differentiate it from the background). The empty field colour is almost independent of the radiation acting on that area of the retina. But it is insufficient to record such differences to fulfil a function which vision clearly does, namely to compare seen and remembered objects (recognition). Here radiations actually acting are compared with those which acted at an earlier time. A system which only recorded differences between two radiations replacing one another would have to store all the radiation changes which have previously been seen; such a system would be very sensitive to disturbances and would require a vast long-duration memory. We may therefore wonder whether there is a special mechanism for comparing radiations seen at different times; two hypotheses, not mutually exclusive, may be proposed. A system whereby all receptors are periodically presented with a standard stimulus (darkness) can effect this function (blinking); only a short-lived memory, of duration exceeding the time between blinks, is then required. In Iarbus's experiments [6] blinking is prevented, so we would expect that only colour differences could be detected; if blinking is replaced by brief extinction of the radiation to the whole retina the vacant field is destroyed.

However, we cannot explain everything by blinking, so a further hypothesis is required. In animals we find not only "on" and "off" receptors, but also ones which give out impulses all the time the light is acting. In animals with very simple visual apparatus (e.g. *Limulus*) the latter type predominates; it is also found in vertebrates.

The small numbers of such receptors prevent their having any great part in forming the image seen, since good resolution is required for this; but even a few receptors giving signals all the time the light acts can equate radiations from memory. This can be seen as follows. Because the eye moves the receptors which record differences indicate the way the colour changes in all directions from a given point (derivative signal). The rare constantly operating receptors give an "integration constant" at points over the field of view. An apparatus of this type corresponding to the retina can easily be imagined to exist in the central nervous system as an integrating system which gives the colour at every point in the field of view.

SUMMARY

The capacity to differentiate radiations is a very important characteristic of any light-sensitive system; this applies not only to animals and man, but also to artificial systems (e.g. a multilayer colour film).

Colorimetric experiments give us this characteristic completely; this is their principal advantage over the threshold method.

In conclusion, the problem of recording the colour at every point in the field of view of the human eye receptors is considered using some experiments by Iarbus with images fixed and moving relative to the retina.

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COLOUR ADAPTATION AND THE LIMITS TO THE APPLICABILITY OF RETINAL RECEPTOR CONCEPTS*

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THE apparent colours of objects are greatly altered by colour adaptation; if a bright red field is viewed for a while, white objects subsequently appear greenish, whereas if adaptation is to a blue-green colour, they appear of an orange tinge, etc. The explanation usually given is that there are three species of receptor in the eye, each with a distinct spectral sensitivity, and each of which species is excited independently of the others and transmits signals to the vision centres. The colour seen is judged

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from the receptor stimuli; colour adaptation produces a differential alteration in general sensitivity between species, so the apparent colour of any object is altered. We have tested the effects observed in colour adaptation against the following explanatory assumptions:

(1) the spectral sensitivity distribution of any species is unaltered by adaptation;
(2) adaptation amounts to sensitivity reduction;
(3) the various receptor species transmit their stimuli to the vision centres independently.

CONSTANCY IN THE SPECTRAL SENSITIVITIES

Colorimetric tests give us the most complete data on the spectral sensitivity curves, which must be compounded linearly from colorimetric combination curves; hence any change in colour matches following adaptation would indicate changes in the spectral curves (or, more precisely, in their linear combinations with one another) or conversely, if no changes occurred. We [1], and Fridrikh in more detail [2], have recently studied colour adaptation effects on colour matches in the Vision Biophysics Laboratory. Our method differs from that of earlier [3, 4] workers in that (a) strictly visual matches are effected, while others, using the Rayleigh equation, have neglected the stimuli to the blue receptors; (*b*) the twilight receptor has been allowed for (four-dimensional colorimetry); (*c*) substitution methods, which automatically ensure that the retina is in the same state for both radiations, are used. The last is particularly important in adaptation studies, since if the retinal state is much altered it is difficult to be sure that the change is everywhere the same, whereas substitution methods avoid this difficulty. We therefore consider Fridrikh's results the most reliable.

We found that colour matches were quite unaffected by adaptation; the apparent colours might alter violently, but the changes were always such that if two radiations were previously indistinguishable they remained so after colour adaptation.* This directly indicates that the retina behaves as a set of receptors for any one species of which we have fixed spectral curves which are independent of the stimuli or states of other species. The retina behaves as a set of independent receptors, but this tells us nothing about whether this independence is preserved in transmission. A different type of experiment is required to solve this problem.

INFORMATION TRANSFER

Suppose that the various receptors transmit their signals independently, and that colour adaptation amounts to sensitivity reduction. The physiological co-ordinates of the apparent colour after adaptation can then only be reduced; in any case, if we use some radiation which does not stimulate some one receptor, the stimulus must still be zero after adaptation. Monochromatic yellow, orange or red lights stimulate the blue receptor only very slightly, so if we are correct in our assumptions we would have that after adaptation, yellow-red colours cannot become blue or purple, since these correspond to strong stimuli to the blue receptor (if the spectral curves are fixed). However, certain data indicate that this is not so; Iustova [5] has found that red becomes of low apparent saturation after adaptation to the self-same red, i.e.

* Fridrikh has also shown that if the twilight receptor is neglected we get a displacement in the equations similar to that found by Brindley [4]. This effect is unrelated to changes in the spectral curves.

that the green and blue receptors are apparently more strongly stimulated. We found that the green and blue receptors are apparently more strongly stimulated. We found [6] that monochromatic yellow appears blue after adaptation to red; but these data are inadequate to show whether the blue receptor is actually more strongly stimulated, or whether the effect is merely due to a drop in the red receptor sensitivity.

To decide this question we used a Demkina colorimeter, at a field diameter of 1·5°, the half-field used for the test colour being first illuminated with the colour used for adaptation. This radiation was then replaced by the test one, the comparison colour being simultaneously cut in on the previously dark half. Since the comparison field could be fed with any mixture of the three basic colours set up in the instrument, the apparent co-ordinates of the test field could be measured. The experiment consisted in selecting amounts of the three basic colours such that at the moment of replacement the colour did not differ from that of the test field. Throughout the tests the observer regarded the dividing line between the fields; the test was repeated periodically, so that the appropriate match colour could be selected. The type of result to be expected if the adaptation is solely due to sensitivity changes is predicted as follows. Let $\bar{r}_0$, $\bar{g}_0$ and $\bar{b}_0$ be the test field co-ordinates, as found without prior colour adaptation (identical adaptation in all parts of the retina); then if the sensitivities fall, the new coordinates must be

$$\bar{r} = \alpha\bar{r}_o, \bar{g} = \beta\bar{g}_o, \bar{b} = \gamma\bar{b}_o \tag{1}$$

where α, β and $\gamma < 1$, and depend on the radiation used for adaptation and the time for which it has acted. No co-ordinate can in any case be greater after adaptation than before.

Tests show that this relationship is not always followed; Table 1 gives the results from one series, all lines relating to the same state of adaptation, which state was ensured by using a standard procedure (standard time and standard orange-red radiation); the reproducibility was checked. The test field was of yellow, of various brightnesses; the apparent colour after adaptation varied from azure to blue to purple, depending on the brightness. The $\bar{Z}$ co-ordinate was increased more than 400 times by adaptation to red light. (Table 1 relates to the subject who gave least response; $\bar{Z}$ increased still more with the other subjects.) It is now common knowledge that the human blue receptor has a curve which practically coincided with the international $\bar{Z}$ curve, so this increase in $\bar{Z}$ cannot possibly be explained by a fall in sensitivity; we must therefore suppose, in order to explain this result, that receptor sensitivities can be raised by adaptation (in this case, the sensitivity of the blue receptor). To test this we used a blue test field instead, other conditions being the same; then $\bar{Z}$ was actually reduced (by about a factor of two). It is therefore impossible to explain these results if we assume the receptors independent, since, using the yellow test field, only the red and green receptors are appreciably stimulated, whereas the apparent colour after adaptation to red is blue. Since the relative spectral sensitivity cannot be affected by adaptation (as Fridrikh has shown), we cannot explain the effects if we assume the receptors to transmit their stimuli independently. Under our conditions the signal resulting from stimuli to the red or green receptors is transmitted in such a form that it cannot be differentiated from that normally received from the blue receptor.

TABLE 1. COLOUR USED FOR ADAPTATION: $3{\cdot}1\times10^4X+1{\cdot}5\times10^4Y+10Z$

Test colour co-ordinates before adaptation			Test colour co-ordinates after adaptation		
$\bar{X}$	$\bar{Y}$	$\bar{Z}$	$\bar{X}$	$\bar{Y}$	$\bar{Z}$
0	0	0	0	0	0
39	36	0·08	22	10	46
74	68	0·15	25	12	69
147	135	0·30	50	39	120
330	302	0·67	58	53	170
930	850	1·9	70	144	210

COLOUR SPACE TRANSFORMS IN ADAPTATION

The transforms implied by (1) cannot describe the changes occurring in adaptation; the difficulty is that these formulae incorporate no terms allowing for the interactions between receptors during transmission to the vision centres,* whereas experiment shows that such interactions occur. Only formulae incorporating interaction terms can be valid; the simplest form one can have is a linear one

$$\begin{aligned}\bar{r} &= \alpha_1\bar{r}_o + \alpha_2\bar{g}_o + \alpha_3\bar{b}_o,\\ \bar{g} &= \beta_1\bar{r}_o + \beta_2\bar{g}_o + \beta_3\bar{b}_o,\\ \bar{b} &= \gamma_1\bar{r}_o + \gamma_2\bar{g}_o + \gamma_3\bar{b}_o.\end{aligned} \tag{2}$$

Here the αs, βs and γs depend on the colour used for adaptation and the adaptation time (but not on the test colour).† Equation (1) is clearly a particular case of

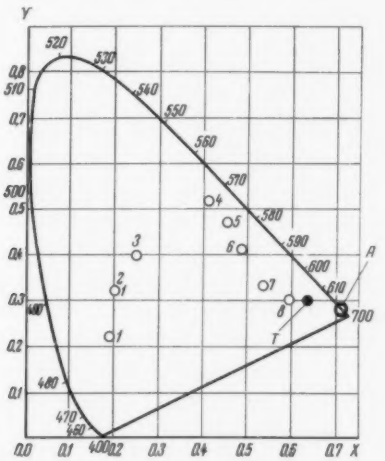

FIG. 1. Change in colour of a test field after adaptation (experiment of Table 2). (A) Colour of radiation used for adaptation; (*T*) colour of test field before adaptation; (*1–8*) colour of test field after adaptation (in order of increasing test field brightness).

* Fedorov's [7] formulae for after-contrast are similarly inadequate.

† The linear formulae can also contain constant terms, in general, but these constants (the after-image co-ordinates for darkness) were not distinguishable from zero under our conditions, without the experimental error. (See the first tests in Tables 1 and 2).

(2), where only α_1, β_2 and $\gamma_3 \neq 0$. The data given in Tables 1 and 2, and in Fig. 1, show that the shift cannot be explained by (2) either, since when the test field brightness (at constant relative spectral composition) is changed, the test field co-ordinates should be reduced in proportion; this does not in fact happen. The co-ordinates vary, but not in proportion to the test field brightness, and their ratios are not constant. A straight line in the test stimulus space becomes a curve in the colour space after adaptation; the transforms by which one passes from one space to the other are nonlinear.

DISCUSSION

We can now establish the extent to which we can say that we have retinal receptors which operate independently and which have fixed spectral sensitivity curves. The stabilities of colour matches in the most varied states of adaptation indicate that the

Table 2. Colour used for adaptation: $340X + 130Y + 0Z$

Test field co-ordinates before adaptation			Test field co-ordinates after adaptation			Three-colour coefficients for the test colour after adaptation		
X	Y	Z	X	Y	Z	X	Y	Z
0	0	0	0	0	0	—	—	—
25	12	2·2	6·4	7·9	20·8	0·182	0·225	0·593
75	36	6·7	10	16·1	23·9	0·200	0·322	0·478
106	50	9·4	16·3	26·4	23·1	0·243	0·401	0·351
220	105	19·6	52	66	8·8	0·410	0·521	0·069
415	198	37	128	132	22	0·454	0·468	0·078
860	410	76	348	297	70	0·486	0·416	0·098
3100	1480	280	2270	1430	540	0·536	0·337	0·127
5900	2800	530	5170	2660	850	0·596	0·307	0·098

relative spectral sensitivities are fixed; any two radiations which produce identical stimuli in one state of adaptation still do so in any other state. Provided that we restrict ourselves to the direct effect of light on the evenly adapted eye, we can without reservation speak of independent receptors; this applies only to radiations producing identical effects on the eye. If tests are done with different parts of the retina in different states, the processes occurring after the light has been absorbed by the rods and cones cannot be represented in independent receptor terms. The signals are transmitted in such a way that the signal received from the blue receptor under some conditions is not distinguishable from that received from the green receptor under other conditions. We therefore consider that when phenomena other than those relating to the direct action of light on the retina are being studied, the independent receptor concept is meaningless; this applies, in particular, to the processes whereby the information is transmitted along the visual nerve. We have shown [8] that single nerve fibres in the frog transmit signals from the two receptors (there are only two in the frog); the possibility of confusion between signals can therefore arise. The fact that such confusion can arise in man indicates that all three daylight receptors exist in a cone, and that they transmit their signals along a single nerve fibre [9].

The above limitations to the receptor concept do not diminish the significance of the Young–Helmholtz theory; they merely indicate bounds to its application. Attempts to overthrow the theory, e.g. by demonstrating that there is an independent yellow receptor, do not derive from colorimetric tests, but from the apparent perception of yellow as a single colour, independent of red and green. No subjective analysis of perceptions can give data on the properties of the receptors, so our restrictions on the receptor concept merely dispose of baseless criticisms.

There is one final observation to be made; in adaptive changes not only do the colour spaces axes alter in direction, but also the alterations are nonlinear. The transform laws depend on how the information is handled subsequent to the receptors, as well as on the properties of the receptors themselves. Until the relevant laws are discovered, adaptation and contrast tests cannot in principle give data on the spectral curves of the primary receptors.

We are indebted to N. D. Niuberg for his constant interest and for his advice.

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SUMMARY

(1) The retina acts as an assembly of independent receptors in its response to light; the independence is preserved only if we restrict ourselves to the light absorption by the receptors.

(2) If any other effects (than absorption) are considered, it is meaningless to speak of independent receptors; this is so, in particular, in relation to information transfer along the visual nerve.

(3) The colour space changes nonlinearly during adaptation, properties other than those of the primary receptors being involved.

(4) Adaptation and contrast tests cannot give the spectral curves of the receptors.

Translated by J. E. S. BRADLEY

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THE EFFECTS OF ULTRASONIC IRRADIATION ON ALIPHATIC AMINO ACIDS*

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CARBOHYDRATES undergo a definite sequence of chemical changes when acted on by ultrasonic irradiation; such changes are also found with the higher alcohols [1]. A typical result is that these compounds give products which absorb at about 265 mμ under these conditions. If alkaline solutions are used, ultra-violet absorption is found at both acid and alkaline pH, the absorption maximum being at 265 mμ in alkali and 245 mμ in acid. These changes in carbohydrates and closely related compounds result in the production of aldehyde groups and of conjugated double bonds.

Aliphatic amino acids also show changes readily detectable by spectrophotometry, when so irradiated, but the effects are more complex and require further investigation.

EXPERIMENTAL

The amino acids used were glycine, serine, leucine, threonine, glutamic acid, methionine, alanine, cysteine, cystine, aspartic acid and hydroxyaminovaleric acid; glutamine and asparagine were also used. All irradiations were done in glass test-tubes in an ultrasonic "fountain", at 740 kc/s and 8 W/cm² of radiating surface. Before and after irradiation they were examined with an SF-4 spectrophotometer. Almost all the aliphatic amino acids used show post-irradiation absorption bonds at 230–300 mμ, the bonds mostly appearing when irradiation and spectrophotometry were carried out at pH ⩾ 13. Table 1 shows that of the dibasic acids, aspartic and glutamic acids are readily altered; even 10 min irradiation at alkaline pH gave a pronounced band with its maximum at 225–258 mμ, rising in height with irradiation time (Fig. 1). Small amounts of ethyl ether (0·2 ml per 5 ml of solution) suppressed the changes (as observed in the spectrum) with aspartic acid (Fig. 1); ether often does this. The changes are also not seen if the aspartic acid is irradiated in acid solution, or in alkaline if the latter is acidified with HCl or other acid immediately after irradiation.

Similar results were obtained with glutamic acid, the changes also occurring on irradiation at alkaline pH, but requiring longer irradiations (60 min) to become appreciable, the maximum being at 260 mμ. An ultra-violet absorption band was also found with asparagine, the maximum being at 272–274 mμ in this case; this band appeared after 30 min irradiation. Glutamine was more stable, 60 min being needed; other peculiarities were also noted, in that the result was independent of the irradiation pH provided the solution spectrophotometered was alkaline.

* Biofizika **3**: 2, 190–196, 1958 [Reprint Order No. BIO 127].

TABLE 1. ULTRA-VIOLET ABSORPTION MAXIMA FROM ALIPHATIC AMINO ACIDS IRRADIATED IN 0·1 N NaOH

Substance	Molarity	Irradiation time (min)	Absorption maximum (mμ)
Aspartic acid ($HOOC—CH_2—CHNH_2—COOH$)	0·0045	10	255–258
Glutamic acid ($HOOC—CH_2—CH_2—CHNH_2—COOH$)	0·07	60	260
Leucine ($(CH_3)_2>—CH—CH_2—CHNH_2—COOH$)	0·08	20	260
Threonine ($CH_3—CHOH—CHNH_2—COOH$)	0·084	60	250
Serine ($CH_2OH—CHNH_2—COOH$)	0·095	20	252–254
Hydroxyaminovaleric acid ($CH_3—CH_2—CHOH—CHNH_2—COOH$)	0·0045	20	265
Alanine ($CH_3—CH_2NH_2—COOH$)	0·10	60–240	No maximum (see text)
Glycine ($CH_2NH_2—COOH$)	0·013	60	250
Methionine ($CH_3—S—CH_2—CH_2—CHNH_2—COOH$)	0·004	60	250–270
Cystine ($S—CH_2—CHNH_2—COOH$ / $S—CH_2—CHNH_2—COOH$)	0·0025	20	Flat maximum (see text)

The hydroxyamino acids were also sensitive; we used serine, threonine and hydroxyaminovaleric acid. In alkali, serine gave a band maximum at 252–254 mμ after 20 min, the band height rising rapidly with irradiation time (Fig. 2). In acid conditions no band appeared, even on subsequently making the solution alkaline. Threonine was more stable, a maximum at 250 mμ only appearing after 60 min, the height increasing with irradiation time, though the minimum rose, which levelled out the curve somewhat.

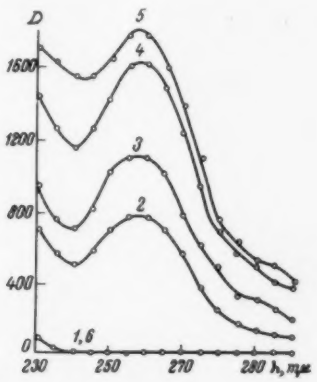

FIG. 1. Aspartic acid, 0·0045 M, solvent 0·1 N NaOH. (*1*) Before irradiation, (*2*) 20 min irradiation, (*3*) 40 min, (*4*) 60 min, (*5*) 105 min, (*6*) irradiation with ether present. Optical density $D \times 10^{-3}$ (same in all figures).

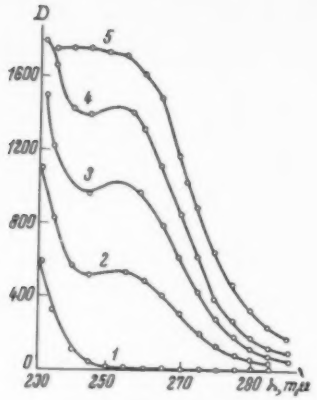

FIG. 2

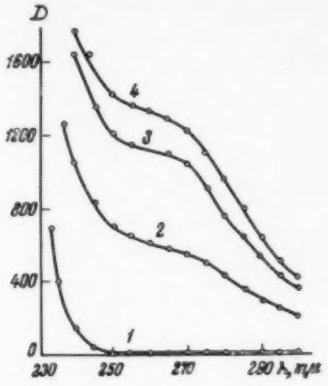

FIG. 3

FIG. 2. Serine, 0·0095 M, solvent 0·1 N NaOH. (*1*) Before irradiation, (*2*) 20 min irradiation, (*3*) 40 min, (*4*) 60 min, (5) 105 min.

FIG. 3. Methionine, 0·004 M, solvent 0·1 N NaOH. (*1*) Before irradiation, (*2*) 60 min irradiation, (*3*) 120 min, (*4*) 180 min.

The sulphur-amino acids showed peculiar changes; on fairly long irradiation methionine gave a flat maximum at 250–270 mμ (Fig. 3). Fig. 4 shows curves for cystine before and after irradiation in alkali, the maximum being flat and at 255–235 mμ at first, the line becoming almost level (minimum lost) after 40 min. Alanine gave an optical density inversely proportional to wavelength; in the 300–240 mμ range the density rose rapidly and continuously on irradiating in 0·1 N NaOH. Glycine gave a different result on irradiation in alkaline solution, the maximum being at 250 mμ (Fig. 5) in alkali and vanishing on acidification, though the optical density of the solution as a whole remained higher than initially.

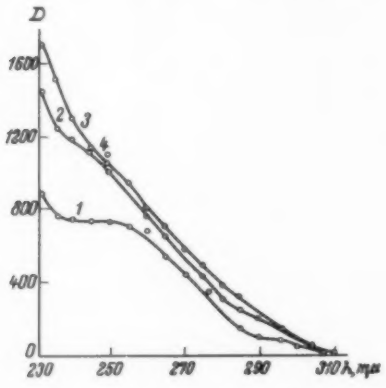

FIG. 4

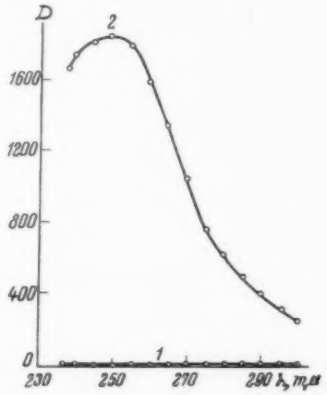

FIG. 5

FIG. 4. Cystine, 0·0025 M, solvent 0·1 N NaOH. (*1*) Before irradiation, (*2*) 20 min irradiation, (*3*) and (*4*) 40 min.

FIG. 5. Glycine, 0·013 M, solvent 0·1 N NaOH. (*1*) Before irradiation, (*2*) 60 min irradiation.

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DISCUSSION

All the aliphatic amino acids used show changes readily detectable by spectrophotometry when irradiated with ultrasonics; only the absorption maxima differ. A common feature is that the ultra-violet absorption bands only appear on irradiation in alkali. Absorption bands in the ultra-violet are also produced by ultra-violet and X-ray irradiation of aliphatic amino acids (aspartic acid, serine, asparagine) in alkaline media, as in our work. Some workers [2] consider that X-irradiation of primary and secondary aliphatic amines in alkaline media containing oxygen produces oximes (RCH: NOH), this occurring via the intermediate peroxy radicals $RCH_2NH_2O_2$, which are reduced as follows at alkaline pH:

$$RCH_2NH_2\dot{O}_2 + O^-_2 \rightarrow O_2RCH{:}\,NOH + H_2O.$$

The O^-_2 is produced by dissociation of HO_2 ($HO_2 \rightarrow H^+ + O^-_2$), this radical being produced by X-rays and ultrasonics. The oximes absorb maximally at 250–260 mμ [3].

Cavitation theory [4] indicates that this mode of oxime formation is fairly likely for aliphatic amino acids also.

The picture becomes more complex, however, if our results on other organic compounds (aliphiphatic acids in particular) are considered; some of these acids also give products with ultra-violet absorption maxima, e.g. succinic, citric and malic (Fig. 6). The other dibasic acids (oxalic, malonic, tartaric, glutaric, adipic) give no effect observable by spectrophotometry even on very prolonged irradiation at acid or alkaline pH. The effects are clearly closely related to the molecular structure, even slight differences having a major effect on the stability to ultrasonics; it is sufficient to compare the data for glutaric and succinic acids (Table 2) to see this. These large differences were not found with the amino acids, the free NH_2 group clearly rendering all the changes detectable in the spectra of the same type. Analogous similar changes occur with succinic, citric and malic acids, but our data are inadequate to indicate whether these acids result in ultra-violet absorbing products the same as with the amino acids. We reject the deamination and decarboxylation hypotheses [5–7], and consider that the effects may be due to nitrogen fixation by the organic compounds;

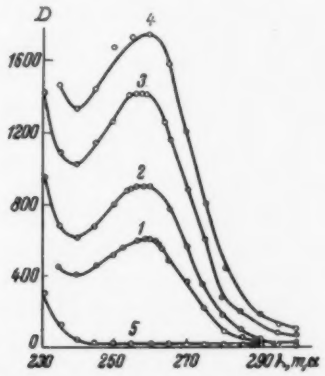

FIG. 6. Succinic acid, 0·042 M, solvent 0·1 N NaOH. (*1*) 10 min irradiation, (*2*) 20 min, (*3*) 40 min, (*4*) 80 min, (*5*) irradiation with ether present, 30 min (also prior to irradiation).

Table 2. Absorption maxima of irradiated aliphatic acids

Acid	Molarity	Irradiation time (min)	Absorption maximum (mμ)
Oxalic (HOOC—COOH)	0·0045	180 (max.)	None
Malonic (HOOC—CH_2—COOH)	0·05	120	None
Tartaric (HOOC—CHOH—CHOH—COOH)	0·07	180	None
Glutaric (HOOC—$(CH_2)_3$—COOH)	0·04	120	None
Adipic (HOOC—$(CH_2)_4$—COOH)	0·07	80	None
Succinic (HOOC—$(CH_2)_2$—COOH)	0·04	10	258–260
Citric (HOOC—CH_2—C(OH)(COOH)—CH_2—COOH)	0·03	10	260–262
Malic (HOOC—CH_2—CHOH—COOH)	0·045	30	260
Formic (H—COOH)	0·21	120	None
Acetic (CH_3—COOH)	0·16	90	None

this may explain why the final products with certain organic acids and amino acids are identical. It has been shown that nitrogen can be fixed by organic acids and other nitrogen compounds formed on ultrasonic irradiation under certain conditions, but the results do not give conclusive evidence on the changes in the amino acids.

Here we only note that the aliphatic amino acids undergo specific changes on ultrasonic irradiation. The absorption bands found are typical of cyclic amino acids, so we may suppose that cyclization may possibly occur with the decomposition products, as well as decomposition involving oxime formation. This is the subject of further study on our part.

SUMMARY

(1) Ultrasonic irradiation produces spectral changes with aliphatic amino acids, ultra-violet absorption bands in the 230–300 mμ range appearing.

(2) These bands appear when the irradiation and spectral examination are done in alkaline solution (pH 13).

(3) They are found with dibasic amino acids, hydroxyamino acids, thioamino acids, etc. Most of them show maxima at 250–260 mμ. In asparagine it is at longer wavelengths (274 mμ); and with methionine a flat maximum is seen at 250–270 mμ. The optical density is proportional to the reciprocal of the wavelength with alanine; a sharp maximum at 250 mμ appears with glycine irradiated in alkaline solution.

(4) Of the aliphatic acids studied only certain dibasic ones (succinic, citric and malic) absorb at 220–280 mμ after irradiation.

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INCREASED RADIORESISTANCE OF *BOMBYX MORI* L. POLYPLOIDS AND THE GENERAL THEORY OF THE BIOLOGICAL ACTION OF IONIZING RADIATIONS*†

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Since the time of the first observations on radiation damage in embryos developing from irradiated ova [1, 2] and on mutations induced by penetrating radiations [3–5], innumerable experiments have demonstrated that the primary effects observable biologically in irradiated cells occur in the nuclei, or more precisely in the chromosome apparatus. In 1947 one of the present authors showed [6, 7] that mulberry silkworm grubs could develop completely and perfectly from eggs given high X-ray doses (up to 550 kr), provided that the original nucleus in an egg was completely excluded from the development, which was completed androgenetically using a nucleus from a non-irradiated egg. But if the irradiated nucleus was allowed to remain, it was found that even at doses a hundred times lower the young showed signs of radiation damage or even death, all other conditions remaining the same.

These observations once again strongly confirmed that the nuclear effects are of particular importance; they also show that the primary changes produced in the extranuclear material (cytoplasm) of the ovum have no harmful biological effect over a wide dose range, if at all; and if they do, they act via secondary changes, induced immediately in the nucleus. The second alternative is contraindicated by the results from certain direct experiments, however [8, 9]; and in essence the second is virtually equivalent to the first, except that the route by which the nucleus is damaged differs.

THEORETICAL ASSUMPTIONS

A survey of all the data [6, 7] enabled us to formulate a general concept: the biological effects of ionizing radiations are related to the primary changes in the nuclei,

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† The data given here were obtained by N. M. Tul'tseva from studies on the change in radiosensitivity with age in silkworm grubs of varying ploidy. As regards the section of special significance in relation to polyploidy it should be considered as a preliminary communication. The work was done to test certain consequences of Astaurov's [6] general theories as to the nature of the biological effects of ionizing radiations.

primary changes in the cytoplasm having no material biological effect. This concept is confirmed by the fact that, apart from a few exceptions dealt with elsewhere [6], practically all biological effects from penetrating radiations (radiation sickness, erythema, skin burns, increased radiosensitivity in malignant tumours or rapidly growing cells, sterilization, depression of regeneration, etc.) originate from primary irreversible damage to the nucleoproteins in the chromosomes. These irreversible changes are reproduced on chromosome division, and if they occupy key positions in the structures responsible for biosyntheses they must disrupt the relation between nucleus and cytoplasm, cellular metabolism, protein synthesis, etc. As the changes are random with respect to the historically developed genotype structure they are largely destructive or damaging. They frequently amount to direct removal or inactivation (via breakage of chromosome links) of vitally important structures.

The biological effect of any ionizing radiation depends little on the radiation type, but much on the properties and subsequent history of the system. If, for instance, a nuclear change in the sex cells is reproduced and extended to all the cells in the individual resulting therefrom, a visible change in phenotype may occur, or development may take a lethal course; we call this a mutation. Large mutational changes (chromosome aberrations) in such nuclei, if reproduced in the gametes, may result in the latter being infertile, or in other forms of infertility, as with silk worms sterilized with X-rays [10]. If the nuclear changes occur in a differentiated tissue which no longer divides (say in a nerve fibre), the change remains localized in the particular cell and, as no effect is exerted thereby on cell division, chromosome reproduction, etc., no effect is seen. But if the change occurs in some rapidly dividing tissue (say, the blood-forming organs or sex cells) the primary effects (chromosome breaks, translocations, inversion, etc.) may become more extensive in subsequent divisions and lead to whole sections or even whole chromosomes being lost, thus in a final reckoning causing extensive damage to the tissues and organs of any offspring, etc.

If the changes occur in resting cells (in seeds, say) the damage is long stored in a concealed form and only appears when development begins, the latent damage then distorting or preventing proper development. The nuclear changes occurring on whole-body irradiation are most varied in type and degree of irreversibility. The primary damage can hinder multiplication or metabolism or even cause cell death; propagating on cell reproduction it may cause secondary functional damage to the organism as a whole. This is our conception of how the effect known by the special term "radiation sickness" develops. If the radiation sickness does not lead on to death, recovery is at best partial and slow (this evidently involving a selection amongst the cells, the most heavily damaged being replaced by the less damaged). This is indirect evidence that radiation reactions involve irreversbile nuclear destruction.

This view has been more fully presented previously [6]; on this basis we see radiation sickness as due to the same nuclear changes as are responsible for mutations, the difference being principally in the system in which the changes occur. It has been shown by complete experimental androgenesis that, in the silk worm, the effects arise solely from nuclear changes, cytoplasmic changes having no effect over a wide dose range; quite independently it has also been found (by the same method, using the *Habrobracon* ichneumon fly) that the cytoplasm is damaged by 15 kr, and that if

50 kr is given to the cytoplasm alone all ova die [11, 12]; but the cytoplasmic effect is here slight compared with the nuclear.

It is at present difficult to say why these different results are found; whether they are due to differences in the properties of the objects, in the stages used or in the methods. It has also been claimed that radiation-induced changes in the cytoplasm are [13, 14] and are not [15] biologically important. Certain data which indicate that primary cytoplasmic damage is important are unconvincing and permit of other interpretations; the problem is as yet unclear and requires further study, and the experimental androgenesis method may here play a decisive part. But at normal dose levels the cytoplasmic contribution to the total biological effect is negligible relative to the nuclear; with this reservation the above concepts as to the nature of the effects of ionizing radiations retain their validity.

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If these general ideas are correct, and any radiation effect, particularly radiation sickness, is due to primary radiogenetic causes, certain consequences allowing of simple experimental test follow. These radiogenetic effects are damaging not because they remove vital cell elements which control processes, but because the elements are quite unique or occur (in diploids) in pairs only. If, moreover, the damage is to unique irreplaceable elements, and thereby paralyzes or impedes vital activities, then if the numbers of such elements are increased, e.g. on going to polyploid systems, the "reserve strength" should increase in the sequence haploid → diploid → triploid → tetraploid, etc., and the radiosensitivity fall correspondingly. This must occur because the probability of simultaneous damage to reduplicated elements must fall in this sequence.

The largest stability gain should be found in going from haploid to diploid forms; this may be why diploid forms are so common in nature. The next greatest gain is between diploid and triploid, subsequent gains being slight. It is therefore clear that if the suggested mechanism is the real one we should find that the degree of polyploidy, frequency of phenotypic mutations and radioresistance ran parallel. There are many facts which confirm this; the fall in the mutation frequency with number of chromosome sets was observed long ago for oats and wheat [16]. More recently it has been found that tetraploid barley, irradiated as seeds, is enormously more resistant than diploid [17]. Mansurova *et al.* have recently obtained good data on diploid and tetraploid buckwheat and millet, the frequency of minute necrotized areas (due probably to somatic lethal dominants) falling almost to zero for the polyploids ([18]; personal communications). Haploid, diploid and polyploid yeast cells show radioresistances in that order, as judged from survival and multiplication data [19–22]. In a similar way it has been found that in male ichneumon fly grubs (*Habrobracon juglandis*) the haploids are far more sensitive to X-rays than diploid females [23]. Here the difference may be ascribed to differential sex resistance rather than to number of chromosome sets, though this could only be proved by using the same sex in both cases [24, 25].

In the case of *Drosophila* no difference in radioresistance has been observed as between diploid and triploid forms, and this, together with certain indirect evidence [26], led even Muller to conclude that radiation damage to developing organisms was physiological (or protoplasmic), and not genetic, in nature [26–28]. This conclusion is clearly directly opposed to our ideas, in which the physiological and genetic effects

are considered as different links in a single causal chain, the genetic effects being the causes of the physiological. The vital importance of this topic has led us to study radiation damage in the silkworm; it is very convenient because parthenogenesis can be produced by thermal means, to give diploid, triploid and tetraploid all-female clones, and by $4n \times 2n$ crosses one can obtain triploids as required by sexual reproduction [29–32].

EXPERIMENTAL

Twenty distinct lines of silkworm were used, 12 of these being parthenogenetic clones (7 were $2n$, 3 were $3n$ and 2 were $4n$) and 8 of sexual origin (5 sets $2n$ and 3 of $3n$). To exclude accidental effects from the particular genetic constitutions we used mixed lines of highly varied natural origin in all cases (inbred lines, first-generation hybrids, etc.). Most of the parthenogenetic clones derived from hybridized females, but some were pure lines. The triploid cross-bred sets (three groups of differing genotypes) were derived by crossing $4n$ females with $2n$ males. In all cases the diapause stage was used, i.e. hibernating eggs at different stages of their ten months development.

The sensitivity was calculated at seven stages of development (series I–VII). Three stages (I–III, ages 10, 60 and 105 days) fell in the summer–autumn rest (aestivation) period, when the embryos were in a profound diapausal state: two (IV, V, ages 150 and 200 days) were in the middle or end of the winter, the temperature being about $+4°C$ (diapause). The two last stages were in the spring development period and were chosen at the middle and end of the incubation phase (VI and VII, 7 and 11 days from the beginning of incubation). Two different series (Va and Vb) were run in stage V; in Va the eggs were put to incubate immediately after irradiation, while in Vb they were returned to the cold store for ten days after irradiation.

The apparatus used was an EUM-3, at 180 kV, 10 mA, no filter, dose rate 99 r/min. In each case an average sample of apparently completely normal and well-pigmented eggs, comprising on average about 250 in all, was used. The index of resistance was the yield of live grubs relative to a control sample incubated simultaneously under the same conditions. The resistance characteristic was taken as the yield of grubs versus dose, all lines being treated simultaneously at doses up to those causing 100 per cent deaths. To determine the limiting permissible doses roughly for 9 lines (7 parthogenetic) we used ones taken at the end of the cold storage period, using doses increasing in geometrical progression from 1 kr, 2 kr . . . 64 kr (control = 0); it was shown that 4 kr was sufficient to produce almost 100 per cent deaths. Assuming the values to be roughly the same for the other stages, we used doses of 0 (control), 500 r, 1000 r, etc., by 500 r to 5500 r (in all, 12 levels) in the main series. In some series lower maximum doses of 4500 r were used (series IV, Va, Vb) and 3000 r in series VI. The 18 lines gave some 1600 samples, each of about 250 eggs.

In drawing up the dose-dependence curves against age the mean yield of the eight control samples in each line was taken as 100, to make the results for different lines (which sometimes varied greatly in absolute yield) comparable. The control yields were found to vary particularly strongly as between parthenogenetic and sexual eggs, being 24–48 per cent in the diploid clones, 12–21 per cent in the triploid and 32–52 per cent in the tetraploid, while the lines gave 72–97 per cent (diploid) and 84–85 per cent (triploid).

The following regularities were found on examining the curves.

(1) There was frequently a difference between parthenogenetic and sexual sets of a given ploidy, the per cent yield mostly falling more rapidly with increase in dose for the former; this difference is probably related to the different yields from the parent stock control specimens. Hence the viabilities of lines of different ploidies were compared for the pathenogenetic- and sexual-origin eggs separately.

(2) All lines varied in viability similarly and regularly during development, the viability falling steadily; certain periods where the viability remained roughly constant could be distinguished. The 10-day specimens of series I were the most viable, the 60-day being less so, while the 105 and 150-day were comparable with the latter (series II–IV). In the next stage (end of cold storage; stages Va, Vb) another fall occurred to a level which remained the same in series VI.

In series VII, which were irradiated only three days before hatching, the viability could not be estimated correctly from the hatching yield, since there was insufficient time for the radiation to have its full effect. Hence the data from series VII were excluded.

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(3) The viability data for a given ploidy again show clearly that the curves for lines of the same chromosome number tend to group together, although the scatter is considerable; also the 3*n* and 4*n* sets, no matter whether parthenogenetic or sexual, give curves lying much higher than for the 2*n*. Not so clearly, but often quite definitely, we see that the 4*n* sets are more viable than the 3*n*, though we must remember that only two 4*n* clones were used, so the mean data might be more distorted by random errors.

As space does not permit us to reproduce the numerous graphs for the various series and lines, we merely give the averaged data for the 2*n*, 3*n* and 4*n* sets for (i)

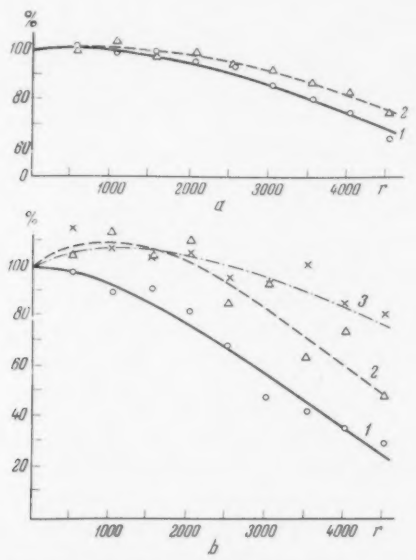

FIG. 1. Relative viabilities (as per cent control) vs. radiation dose (in r) for young of types 2*n*, 3*n* and 4*n*. Averaged data from the II, III and IV age series. (*a*) young of sexual origin; (*b*) parthenogenetic young; (*1*) diploids, (*2*) triploids, (*3*) tetraploids.

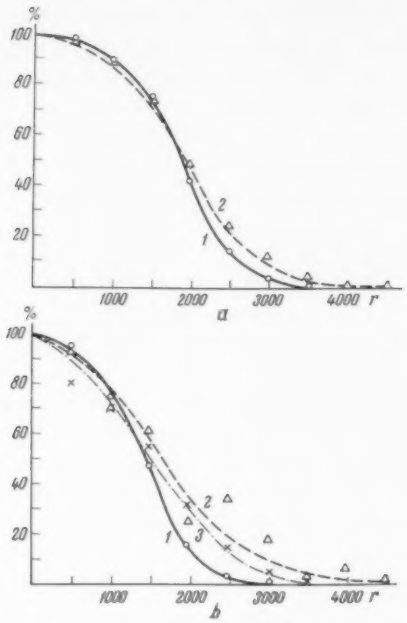

FIG. 2. Relative viabilities (as per cent control) vs. radiation dose (in r) for young of types $2n$, $3n$ and $4n$. Averaged data from the Va, Vb and VI age series. (*a*) young of sexual origin; (*b*) parthenogenetic young; (*1*) diploids, (*2*) triploids, (*3*) tetraploids.

series II–IV and (ii) series Va, Vb and VI (Figs. 1 and 2). Since within each of these groups the viability was roughly constant the various series can be considered as duplicates (II–IV in the first case, Va, Vb and VI in the second).

Fig. 1 shows the relation of viability to dose for case (i), separated into sexual (*a*) and parthenogenetic (*b*) samples, Fig. 1 (*a*) showing 5 diploid sets (i.e. the mean of 15 experiments) and 3 triploid sets (9 experiments). Fig. 1 (*b*) for the $2n$ clones is the mean of 15, for the $3n$, the mean of 9 for the $4n$ and the mean of 6; Fig. 1 (*a*) shows the advantage of $3n$ over $2n$ clearly while Fig. 1 (*b*) shows that the sequence is $2n \rightarrow 3n \rightarrow 4n$. Fig. 2 for series Va, Vb and VI is analogous, (a) being the sexual sets, being the mean of 13 experiments with the $2n$ samples, and of 7 for the $3n$, while (b) is the mean of 15 for the $2n$, 7 for the $3n$ and 6 for the $4n$. Here again the polyploids are systematically more viable than the diploids, though the parthenogenetic tetraploids are not so viable as the triploids, which may be, as we have said, accidental, because we only had two $4n$ clones.

CONCLUSIONS

The radioresistance of silkworm embryos in the stages from diapause to middle spring development increases with the degree of polyploidy, other things being equal. There is a marked rise in resistance between diploid and triploid, and a lesser one between triploid and tetraploid. The data support the ideas as to the essence of the biological effects of ionizing radiations proposed in the first section; they show that the main causes of such effects as radiation damage to embryos of multicellular organisms, are genotype changes in the cell nucleus.

Translated by J. E. S. BRADLEY

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FORMS OF INACTIVATION OF YEAST CELLS BY IONIZING RADIATIONS*

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WE have previously shown that the degree of inhibition of the first post-irradiation division is related to dose to the yeast cells in a way different from that for irreversible loss of the power to multiply indefinitely. We therefore concluded that inhibition of the first division is not the end-effect, but just one of the first steps in a radiation after-effect which results in subsequent inactivation [1, 2]. It was thus necessary to

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establish at what time the irradiated cells finally lost the power to multiply, i.e. to find the time variation of the radiation after-effect.

Although many workers have described delayed radiation inactivation in yeast cells [3–7], the quantitative aspects have been largely neglected; in particular, data on the number of division cycles preceding inactivation are lacking. It has also not been established whether the inactivation can be reversible, or how the various types of radiation damage are related to dose; these problems are directly related to the mechanisms by which ionizing radiations produce their effects. It is of fundamental importance also to establish how the type of inactivation is related to polyploidy, to which some workers [6, 8–11] assign a decisive role in radiobiology.

METHODS

We used diploid *Saccharomyces vini* cells, Megri-139-B strain, haploid *Zygosaccharomyces Bailii*, obtained from Professor Kudriavtsev, and a haploid strain XII of brewer's yeast, derived from a diploid strain and kindly presented to us by Professor Kosikov.* Forty-eight-hour cultures were grown at 30°C on a solid medium, suspended in sterile tap-water and γ-irradiated by a GUT-Co-400 machine, the cell concentration being about $0{\cdot}5 \times 10^6$/ml, with the fraction of dividing cells not over 0·1–0·2 per cent, dose rate 1070 r/min, and the doses given varying from 7·5 to 888·5 kr.

The main data were derived by examining fixed preparations, made as follows. A drop of the suspension was spread on a glass slide with a spatula and covered with a thin layer of nutrient agar; the preparations were incubated as required in a humidified chamber at 30°C and then treated with formaldehyde vapour and dried at 45–50°C. The microcolonies preserved their shapes well, the individual cells being clearly visible; the preparations could be used unstained at ×100 or ×200. The numbers of colonies containing various numbers of cells were determined after different incubation times, 1000–1500 microcolonies being studied at various points in the preparation. Groups consisting of 1, 2, 3, 4, 5, 6, 7–12, 13–24, 25–48, 49–99, 100–200 and over 200 cells were assigned to different classes; the latter six thus cover colonies resulting from three or more division cycles.

We deliberately used non-dividing cells uniformly spread over the medium as single cells, and a large number of cells, to ensure accurate results; the results did not usually vary more than 3–5 per cent between parallel sets, or 10 per cent between repeat sets. In this way we derived a clear idea of how the microcolonies grew in the control and irradiated preparations, and were also able to observe the various forms of inactivation.

I. THE POST-IRRADIATION CHANGES IN DIPLOID YEAST CELLS

We used the *Saccharomyces* strain to derive the quantitative laws of post-irradiation change; Fig. 1 shows the growth curves. The control cells began to bud after about 60–70 min at 30°C, all cells soon dividing and no single cells usually being left after 4 hr (Fig. 1(1)). A second cycle began when the first had finished (Fig. 1(2)), two-cell groups being replaced by sets of four (sometimes three), the pairs going over to

* We are indebted to Professors Kudriavtsev and Kosikov for their valuable advice and assistance in this work.

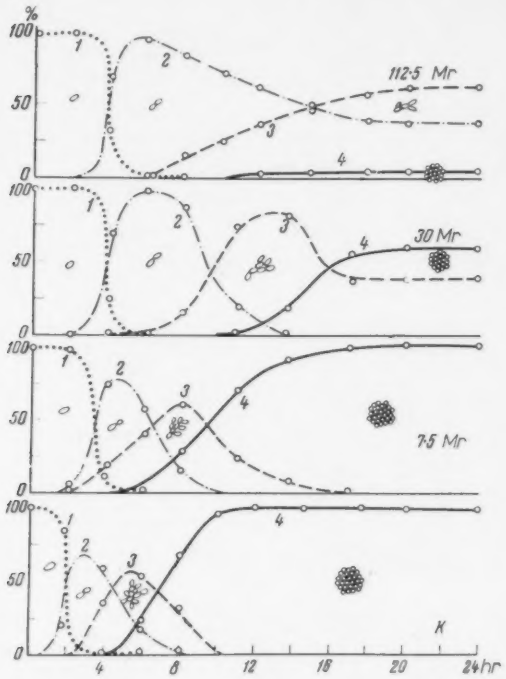

FIG. 1. Changes in microcolony forms on 24 hr incubation at 30°C. Distributions of the various forms as percentage of total colonies vs. radiation dose. (*1*) single, (*2*) pairs, (*3*) chains, (*4*) disks. K = control.

chains, which then branched (Fig. 1(3)). After 3–4 cycles disk forms typical of normally dividing yeast cells appeared (Figs. 1(2) and 2(1)). Further growth up to macrocolony size occurred in the disk form.

The division process took a different course in the irradiated cells, the first division being delayed but not inhibited up to 350 kr. All cells showed this delay, as when liquid media are used [2], the time it lasted being not more than 2 hr, a dose saturation effect being observed. The second division was also delayed, the length of the delay rising rapidly with dose and varying widely between cells (Fig. 1(2)). This effect was much less reversible.

After the second division some 2–5 further divisions occurred at a reduced rate, some colonies then beginning to divide at the normal rate while others showed a third depression in the growth rate (Fig. 1(3)). The fraction of cells which failed to reproduce further after 4–7 cycles varied with dose, this remote inactivation being almost completely irreversible. Three depression periods are thus seen when solid media are used; the first covers all cells during the first division, lasts not more than 2 hr and is reversible in all cases at doses below 350 kr; the second also covers all cells at the second division, its duration increasing with dose and the fraction of cells recovering falling rapidly; the third occurs after 4–7 cycles, covers only part of the cells and is irreversible.

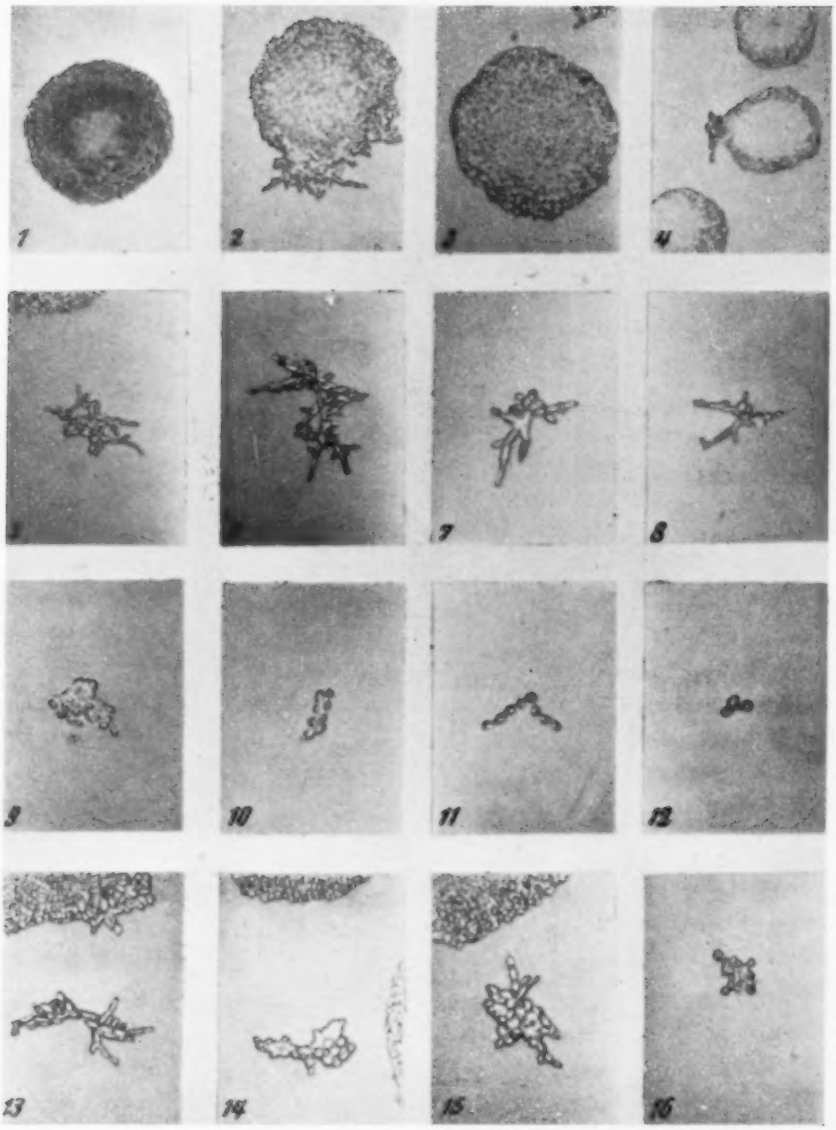

FIG. 2. Twenty-four hour microcolonies of yeasts of varying chromosome make-up. Diploid yeasts, dose 30 kr: *1*—normal colony, "disk" (magnification 10×7); *2*—recovering forms (magnification 20×7); remotely inactivated forms "chains" *5* and *6* (magnification 20×7); *7* and *8* (magnification 40×7). Natural haploid yeasts, dose 15 kr: *3*—normal microcolony (magnification 20×7); *9–12*—remotely inactivated forms (magnification 40×7). Artificial haploid yeasts, dose 15 kr: *4*—recovering forms (magnification 10×7); *13–16*—distantly inactivated forms.

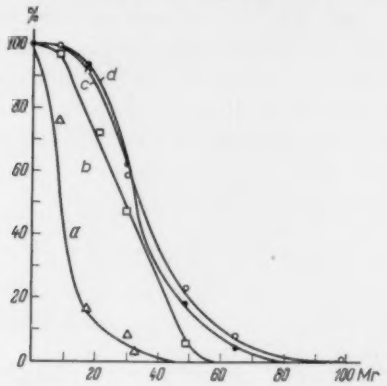

FIG. 3. Percentage of disks vs. radiation dose after various incubation periods. (*a*) 12 hr, (*b*) 18 hr, (*c*) 24 hr, (*d*) survival curve found by the standard method of counting macrocolonies in Petri dishes.

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These post-irradiation changes agree well with our earlier [1] observations on the growth of macrocolonies from irradiated cells. The differences between control and irradiated cultures are most noticeable at short incubation times, becoming less evident as time passes and reaching a steady level after about 20–24 hr at 30°C (Fig. 3). Fig. 3 indicates that the microcolonies are only inactivated at stages preceding disk formation, the disk forms being typical of healthy, normally growing cells. Reactivation in irradiated cells is practically complete within 24 hr at 30°C, and microcolonies which have not been transformed into disks by this time soon die.

Fig. 4 shows how the colonies which cease to grow are disturbed over the various classes after different numbers of cycles. The characteristic relationship to dose confirms our idea that there are three distinct types of death to which the irradiated cells are subject. At low doses ($\sim 10^4$ r) most cells lose the power to divide after 4–7 cycles; this delayed inactivation occurs in groups consisting of 16–150 cells, loosely packed and containing hypertrophied individuals (Fig. 2(5–8)). The maximum for

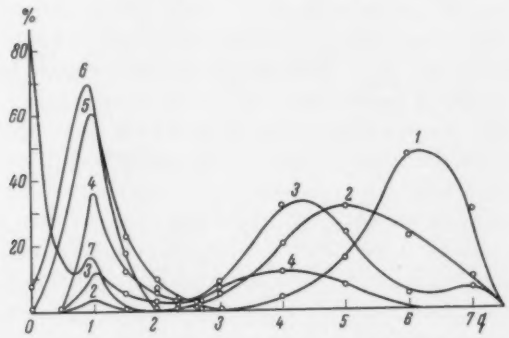

FIG. 4. Inactivated microcolonies (as percentage of total dead cells) vs. mean number of divisions (*q*) of the original cells for various radiation doses. (*1*) 16 kr, (*2*) 49 kr, (*3*) 65 kr, (*4*) 97 kr, (*5*) 162 kr, (*6*) 432 kr, (*7*) 639 kr.

this form is reduced in height and shifted somewhat to lower doses as the dose is increased, while the number of colonies inactivated at the two-cell stage rises rapidly. At still higher doses the cells are inactivated, without division. Fig. 5 shows how the proportion of each form varies with dose; the damage curves obtained by counting macrocolonies on Petri dishes are clearly the sums of three curves of the type shown here, each of which has a definite dose threshold.

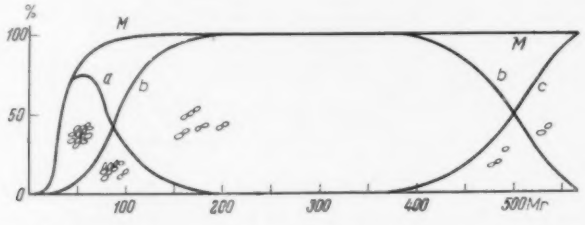

FIG. 5. Forms of inactivation in relation to dose. *M*, total damage curve; (*a*) inactivation after 4–7 divisions, (*b*) after 1 division, (*c*) without division.

2. EFFECT OF POLYPLOIDY ON POST-IRRADIATION EFFECTS

It has been claimed in the radiobiological literature [8–10, 12] that the reactivation and late death effects are closely related to the ploidy, occurring only with diploid or polyploid forms. This would imply that haploid strains would die without prior division or after one division only, no recovery effect being found. Studies on microcolonies produced by 20 hr of incubation at 25°C using X-irradiated diploid and haploid strains of *Saccharomyces cereviseae* have led to this conclusion [8]. It has also been shown that while the sizes of diploid colonies after irradiation differ considerably from the controls, the haploid show no such effect [12]. The relation of type of inactivation to ploidy has usually been considered a proof that the lethal radiobiological effect of radiation is genetic in nature [8–10].

To check this we studied natural and artificial haploid yeast strains (the latter derived from a diploid strain). It was to be supposed that the precise laws of post-irradiation development (survival curve, growth rate, relationship of inactivation forms, etc.) would be different in the haploid forms, but nevertheless we only studied the qualitative aspects, as these are of major importance. Fixed preparations were used, at doses from 7·5–60 kr, and multicellular inactivation forms were demonstrated in both our haploid strains; at mean lethal X-ray doses the cells frequently died after 3 or more divisions, as microcolonies (Fig. 2(9–16)), as in the diploid forms (Fig. 2(5–8)), though these microcolonies usually consisted of less individuals than with the diploid strains. The recovery figures for the haploids were also studied; the late reactivation forms are easily differentiated from normal ones by the shapes of the chains or bundles of cells, which are hypertrophied on reactivation and lie on the edges of the disks formed by their descendants. In the diploids reactivation mostly occurs after the first division, depression occurring after 4–7 cycles usually being irreversible; sometimes recovery to normal occurs after a fairly long depression period (Fig. 2(2)), but the numbers are very small. In the haploid strains, however, recovery after 3–4 cycles is much more frequent.

We may wonder why this inactivation after several divisions in haploid strains had been missed by previous workers; the cause should be sought in the methods used. Thus, Gunter and Kohn [12] studied the dimensions of macrocolonies and found no relation to irradiation for haploids because the post-irradiation depression is brief in cells not inactivated, haploid cells showing a much shorter delay in the first division than do diploids, although they are more radiosensitive [7]. As regards the results of Laterjet and Ephrussi [8], we explain these as due either to the physiological peculiarities of the cells (they irradiated growing cells) or to the X-ray doses they used. Fig. 5 clearly shows that at high enough doses even diploid cells die without dividing, or after dividing once; haploids are more radiosensitive [11] and so the inactivation pictures obtained at identical doses should differ.

DISCUSSION

All yeast cells are damaged to some extent by ionizing radiations; this appears clearly even at low doses in a delay in division in all cells. At higher doses the damage takes on a cyclic character, periods of depression being followed by periods of recovery. After long incubation (48–96 hr at 30°C) the reversible disturbance to the division rate is observable macroscopically as a reduction in disk diameter and an increase in the scatter [1]. The three depression periods correspond to three definite types of death, there being three inactivation peaks, the times of onset of which scarcely depend on dose, dose only affecting the relative proportions of the forms.

The same radiation effects are found in vertebrates [13, 14], insects [15], coelenterata [16], the lower vermes [17] and in other objects [18, 19], wave-like maximum death periods and damage forms occurring at more or less fixed times. Only certain infusoria [20], of single-celled forms, have so far been sufficiently carefully studied, the data agreeing well with ours. Many papers also contain details which indicate that this wave-like variation in radiation damage is very common in unicellular organisms. Thus, late death has been described for flagellates [21] and algae [22], inactivation for bacilli at the multicellular microcolony stage [23] and late effects in *Azotobacter* and related bacteria [24]. The data on the periodic radiosensitivity variations in irradiated *Paramecium* are extremely significant [25].

This wave-like effect is not only produced by ionizing radiations, since *Paramecium* shows a similar response to ultra-violet light [26–28]: certain chemical agents can also cause late effects. Thus, when an animal infected with malaria is injected with chloridine the plasmodia only die a considerable time after entering the mosquito, at the early division stages of the sexually produced zygotes.* Late damage effects after brief exposure to methylene blue [26] or certain other substances [29–32] are known in infusoria.

The time variations in radiation after-effect and the dependence of inactivation form on dose make it very difficult, if not impossible, to explain the late deaths of single-celled organisms in terms of mutations, as Atwood and Stapleton [33] do; Kimball [20] has stated that mutations of unknown type which can affect unicellular organisms in such a way that at first partial or almost complete recovery occurs, followed by death after 4–10 cycles, are unknown in genetics. Our data on the inactivation forms of natural and artificial haploid yeast strains which are multicellular

* Personal communication from Professor Sh. D. Moshkovskii, to whom I am deeply indebted.

would strongly contraindicate a genetic explanation. It would be more logical to suppose that the cause of late death is that several processes are initiated by the radiation which involve different components of the cell and different kinetic laws, thereby giving rise to the wave-like damage variation. Then the delay in division would indicate that the processes have reached some threshold level, while recovery would indicate that they have died away, and any changes produced have been restored.

In relation to the nature of such processes we have previously observed [34] that the kinetic aspects of radiation after-effects in unicellular organisms do not conflict with Tarusov's proposed chain mechanism [35], although this does not, of course, rule out other explanations, if more applicable. As yet little has been done on the time variations in damage to such organisms, and it would be premature to construct more definite hypotheses as to the mechanisms.

SUMMARY

(1) Yeast cells incubated at 30°C on solid media after γ-irradiation show three periods of delay in division which differ in the way they vary with dose, in reversibility and in distribution within the irradiated population.

(2) Three types of inactivation are found: without prior division, after 1 (rarely 2) and after 4–7 divisions. The distribution of these forms over the population depends on the dose.

(3) The late inactivation effect (after several cycles of division) and recovery are not directly related to the ploidy and are found in haploid and diploid strains, at least if resting cells are γ-irradiated.

(4) It is supposed that the wave-like development of damage is a feature of the reaction to ionizing radiations shown by many unicellular organisms, as well as by multicellular ones.

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THE MECHANISM OF THE OXYGEN EFFECT IN RADIOBIOLOGY*

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Many workers have demonstrated that oxygen affects the damage from ionizing radiations experienced by very various living objects and biologically important materials (proteins, nucleic acids, etc.). The oxygen effect is taken to prove that the radiation action is indirect. The mechanism, on the view generally adopted, consists in the oxygen accentuating the damage by producing new free radicals, i.e. by altering the products resulting from the radiolysis of water.

Here we attempt to demonstrate that there is another, essentially different mechanism for the oxygen effect. This derives from certain concepts of the primary radiation action mechanism previously put forward by one of the present workers [1]. It was supposed that some of the energy absorbed in damaging the object and responsible for the physico-chemical after-effects is conserved in long-lived excited electronic states in the macromolecules.

The observation by Gordy *et al.* [2] (using electron paramagnetic resonance—e.p.r.) that many unpaired electrons persist in irradiated objects confirmed this idea. It was also found [2] that the e.p.r. spectra of irradiated biological materials were only retained for long periods if the materials were *in vacuo*; if oxygen was present the lines vanished or altered. As we consider the unpaired electrons retained in irradiated objects as latent damage, while oxygen, as the radiobiological literature would imply, has a damaging effect, it is natural to wonder whether oxygen acts by reacting with the irradiated system.

* Biofizika **3:** 2, 215–219, 1958 [Reprint Order No. BIO 130].

This paper is concerned with demonstrating such a mechanism. On the above grounds we suppose that (*a*) the oxygen acts by reacting with the irradiated system, and not (or not only) by participating in the radiolysis of water; (*b*) the degree of damage due to oxygen depends on the dose, being due to the latent damage stored in the object. To prove this, experiments in which the oxygen damage during and after irradiation can be strictly differentiated are required.

The protein myosin was used for the purpose; its radiosensitivity had previously [3] been studied and a radiation after-effect demonstrated. The solutions used had a concentration of 4 mg/ml and were X-irradiated in the cold (80 kV, 20 mA, filter cuvette bottom, 1 mm Perspex)*; they were first de-aerated on a fore-vacuum pump. Some hours after irradiation the solutions were again saturated with air and the protein stored for a long time in the refrigerator. The ATPase activities of the control protein (M), of the vacuum-irradiated protein [M_v] and of the protein irradiated in air [M] were compared. The main experiments were done at 50 and 25×10^3 r, corresponding to 48 and 30 per cent inactivation.

The ATPase activity was determined by incubation with ATP in borate buffer (pH 9·2) at 37°C for 10 min. In the case of the protein irradiated *in vacuo* the

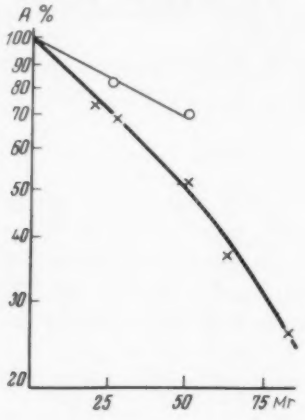

Fig. 1

incubation was also done in air. The effect of atmospheric oxygen, which may inactivate the myosin during the incubation, was allowed for as follows. A small amount (0·2 ml) of the solution was rapidly mixed with a relatively large (~4 ml) volume of reagents previously freed from dissolved air. In most cases the air did not have time to come back into the solution during the incubation, the activity remained high, and so at about 5×10^4 r (myosin about one-half inactivated in aerobic conditions) the first specimen gave 95–97 per cent of the control activity in some cases, although the activity dropped rapidly as the air entered the de-aerated cuvette, the drop

* The irradiation was done on apparatus at the Institute of Physical Chemistry, Academy of Sciences of the U.S.S.R.; the authors are indebted to their colleagues at this Institute for their assistance.

occurring in a time comparable with the incubation time. Subsequent specimens, taken 1–2 min after opening the cuvette, showed lower activities than the first.

The irradiated protein thus rapidly captures oxygen, which reduces the activity. The true activity of the vacuum-irradiated protein was established as follows. The first specimen activity $[M_v]_1$ was determined as above and was compared with that for the same protein incubated with reagents previously saturated with oxygen (by bubbling); this gave $([M_v]+O_2)$, which was very different from $[M_v]_1$ (oxygen step). The less this latter differed from (M) the larger the "oxygen step", the activity then reaching a definite level (Fig. 2). At 5×10^4 r this ATPase activity level was 71·5 per cent of the control, this value being retained for many days subsequently (the arrow in Fig. 3 denotes where O_2 was admitted). This level was higher than for material irradiated in aerated solutions (Fig. 1; the upper curve is for this case).

Check experiments showed that oxygen present during the activity determinations had no effect on the activities of control material or material irradiated in aerated solution. The data indicate that the ATPase activity is unaffected by irradiation in de-aerated solutions, but if oxygen is subsequently allowed in, rapid inactivation down to a certain fixed level occurs (Fig. 3). The same sort of result was found at half the dose (Fig. 3b), the protein irradiated *in vacuo* being about as active as the control,

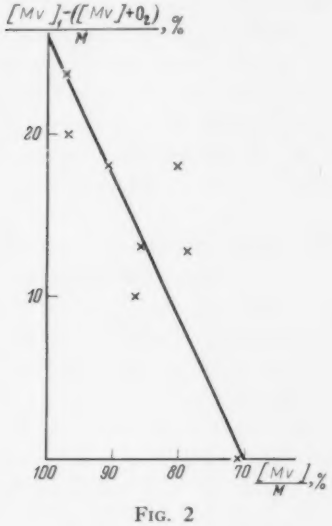

FIG. 2

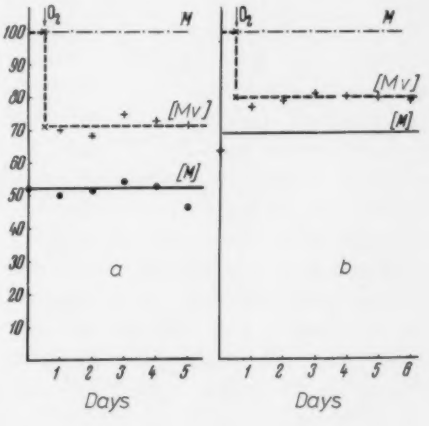

FIG. 3

while that irradiated in aerated solutions gave a very different value. The "oxygen step" was again present, the steady level (~80 per cent) being again above that for material irradiated in aerated solution. The degree of inactivation by oxygen thus clearly depends on the dose level. At higher doses ($80–180\times10^3$ r) the material irradiated *in vacuo* could not be obtained with an activity close to the control under our conditions, although the aerated solution value was always exceeded (*e.g.* the ordinary oxygen effect was present) and a drop always occurred on admitting oxygen.

DISCUSSION

The data indicate that the radiation inactivation of myosin is a two-step process. When the radiation energy is absorbed some sort of excited state is produced in the protein, which state does not involve loss of ATPase activity (stage one); this state is produced by *in vacuo* irradiation. The excited state persists on storage *in vacuo* for a long time, though the protein, while retaining its activity, is definitely altered, as its ready subsequent reaction with oxygen indicates. This reaction constitutes the second stage, the enzyme being thereby inactivated to an extent dependent on the radiation dose.

From the e.p.r. standpoint stage one corresponds to the unpaired electrons being retained and stage two to their loss, consequential on admitting oxygen. This mechanism for the oxygen effect on myosin is in no way a special case, but is a general feature of the oxygen effect. This interaction with excited molecules may be supposed to occur with radiation-inactivated enzymes and other objects showing the oxygen effect.

Some other similar work [4, 5] in which analogous conclusions are reached has recently been published; Alexander [4] has found an oxygen effect when fast electrons or γ-rays act on dry typsin preparations, the inactivation with oxygen present being 30 per cent greater than if it was absent. Alexander concludes that the oxygen effect occurs otherwise than via the radiolysis of water; we find the same with our enzyme. Because of the special technique used (irradiation and action of oxygen occurring at different times) we were not only able to show that a fresh mechanism occurs but also to differentiate the two stages. Alexander's results relate to the second stage and correspond to the upper curve of Fig. 1 in our experiments; the long-lived excited state could not be observed by Alexander's method. Since the new mechanism has been observed with two completely different enzymes one may suppose that it may be general to enzyme inactivation processes.

However, its generality may be much greater; Glegg's [5] results, obtained by a technique rather similar to ours, indicate that, in the radiation depolymerization of wood cellulose, oxygen still has an effect after an irradiated object has been stored *in vacuo* for a long time. The rapidity of the reaction with oxygen ("oxygen step") was not studied, the study being limited to the variation in the after-effect in prolonged experiments, but the similarity between the mechanisms in processes so different as the inactivation of myosin and the depolymerization of cellulose cannot be accidental.

The oxygen effect mechanism may also play a large part in normal enzymatic processes; Commoner *et al.* [6] have shown by e.p.r. methods that actively metabolizing tissues (green leaves, liver, kidneys, etc.) show high unpaired electron concentrations in normal conditions. The free radical concentration in dry yeast cells also increases sharply on activating the metabolic processes, the radicals being retained for long periods *in vacuo* but vanishing in air. The radical production rate may be supposed related to the intensity of the enzymatic processes, while the oxygen effect may have the same mechanism as above.

Our experiments, while indicating a new mechanism for the oxygen effect, do not rule out the possibility that oxygen also plays a part in the radiolysis of water; Fig. 3 shows that the activity of protein irradiated *in vacuo* and then acted on by oxygen is

different from that for material irradiated in aerated solution. The difference is due to the action of oxygen during the irradiation, which may be due to the oxygen influencing the radiolytic products from water and producing long-lived free radicals; the degree of excitation of the protein would then be greater and the dissolved oxygen would have a greater effect. However, the difference in the activities may also be explained without invoking oxygen effects in the radiolysis of water. The inactivation of excited molecules may be reduced by recombination processes which are interfered with when oxygen is present during the irradiation.

The mechanism whereby the oxygen reacts with excited myosin molecules and inactivates them is as yet not clear; whether the oxygen acts as an electron acceptor, or whether the high paramagnetism of the O_2 molecule is responsible, is a subject for further study.

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THE EFFECT OF WHOLE-BODY X-IRRADIATION ON ABSORPTION PROPERTIES OF THE LIVER*

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IT has been shown in a previous communication [1] that radioactive colloids produce changes in the absorption properties of rat liver. Within 2 hr after whole-body irradiation with 1000 r a marked decrease of liver absorption was found as compared with normal controls; within 24 hr the absorption of colloids was at a minimum. In the course of 48 hr absorption increases again, approaching normal values in quantity, and sometimes exceeding control values. These changes were found after i.v. injection of colloidal chrome-phosphate (^{32}P), of iodinated gamma-globulin (^{131}I) or of colloidal Ag (^{110}Ag), as well as with perfusion of a surviving liver preparation with colloidal ^{110}Ag.

This decrease in absorption was measured by comparing the activity of the perfusate with the activity of dried tissue preparations from liver. In previously published work [2–4] an attempt was made to show that the course of the first 24 hr involves processes of depolymerization in the irradiated tissue, in which macromolecular nucleic acid polymers, nucleoproteins, glucoproteins of the hyaluronic

* Biofizika **3:** 2, 220–225, 1958 [Reprint Order No. BIO 131].

acid type, etc., are broken down. In view of these findings an assumption has been put forward [1] that changes in native macromolecules could be the cause of changes in the physical—chemical properties of the cell surface (above all in the reticulo-endothelial system), of their cytoplasm and microstructures, thus affecting the adsorption properties of colloids. In order to test this hypothesis the distribution of applied colloids in tissue microstructures was examined, along with changes in this distribution, under the influence of irradiation. Histochemical and histoautoradiographic techniques were used.

METHODS

Surviving rat liver preparations, either in normal or post-irradiation animals, were perfused with colloidal ^{110}Ag by a previously described method [1]. After saturation of the liver with Ag, and subsequent perfusion with Ringer-Locke solution, in order to remove free (non-adsorbed) colloidal Ag, the liver was fixed in a mixture of 96° ethanol with formaldehyde (10: 1). For fixation, pieces of tissue several millimetres thick from various areas of the liver were used, dehydrated in various alcohols and benzene, and finally fixed in paraffin. A series of slices about 6 μ were stained with haematoxylin. It was possible to see aggregates of coagulated Ag in the form of sharply delineated black grains and plaques.

Tissue was prepared for microhistoautoradiography in the following way: pieces of tissue about 1 mm thick were fixed in cold 130° iso-octane. Frozen tissue was vacuum dried at −40°C. Saturation of the dried tissue with paraffin was carried out in the same apparatus. Very thin layers of a light-sensitive emulsion were spread on microscope slides by submerging them in the fine-grain nuclear emulsion NIKFI [6] (the layers were 2–3 μ thick).

Wetted paraffin slices were then placed on the slide. After 45 days of exposure, the slides were deparaffined in xylol, and then developed, dried, and the resulting microradioautographs fixed in Canada balsam. Control preparations containing no activity showed that the chemical effect of tissue on the photo-emulsion was not present under these circumstances.

The technique described above ensured preservation of the radioactive indicator in tissue slices, as well as a high degree of resolution in the resulting films.

All experiments were carried out with male white rats, of weight 230–250 g. Irradiation was carried out with an X-ray apparatus (voltage 180 kV, current 30 mA, filters 0·5 mm Cu + 0·75 mm Al, dose rate 43 r/min). Results obtained after whole-body irradiation with 1000 r are presented.

RESULTS

Examination of normal liver histological preparations, some of which are shown in Figs. 1(*a*) and 1(*c*), revealed phagocytosis of Ag in the form of black grains in Kupffer cells, deposition of Ag on capillary walls, and adsorption on endothelial cells of large blood vessels. While the absorption of colloidal Ag by Kupffer cells is a general and well-known phenomenon (a sign of reticulo-endothelial activity) the adsorption of Ag on the endothelium of capillaries, and particularly in peripheral and central veins, deserves special attention. It is worth mentioning that the vascular compartment of the liver was thoroughly washed of Ag suspension previous to fixation, so that in the microscopic preparations the visible particles represent those which are firmly fixed

on to cellular elements. It was possible to find in histological preparations only the coagulated portion of colloidal Ag; the autoradiograph gives a picture of total Ag distribution as it adsorbed in the liver. As can be seen in Fig. 1(*b*), an autoradiograph of a normal liver slice, there occured a considerable aggregation of colloidal Ag on the walls of both large and small veins, in capillaries, and in numerous Kupffer cells. In comparing autoradiographs with normal histological slices, the reduction of Ag grains in the emulsion may be produced by a radial dispersion of emission from ^{110}Ag, and this fact must be taken into account. A point source of ^{110}Ag in the tissue would therefore produce a larger area of reduced Ag in the emulsion. Localization of colloidal Ag, as shown by autoradiography, therefore appears to be quantitatively more widespread than it actually is, even if essential histological structure is preserved.

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Examination of analogous preparations 2 hr after irradiation (cf. Fig. 2*a*, *b* and *c*) revealed a marked decrease of Ag in both histological preparations and autoradiographs. Black Ag grains were absent in the histological preparations, and only brown grains were found in Kupffer cells; deposition of Ag on the endothelial surface of large veins was practically absent. The distribution of adsorbed Ag 2 hr after irradiation differs primarily in localization and in the diffuse adsorption of finely dispersed particles by capillary endothelium, as contrasted with the original concentration of Ag aggregates in Kupffer cells and their branches. Consequently, X-irradiation altered adsorption by capillary endothelium on a background of an initial decrease in phagocytosis by Kupffer cells, which was apparently associated with a change in physico-chemical properties of cells. The absorbed Ag was deposited in the form of yellow-brown grains rather than black aggregates, i.e. it was less coagulated and reduced.

Even more marked changes can be found in liver slices perfused with colloidal Ag 24 hr after irradiation (Figs. 3*a*, *b* and *c*); the endothelium of large veins had completely lost its capacity to adsorb colloids. This sharp change in adsorption by endothelial cells was particularly distinct when autoradiographs of irradiated and non-irradiated tissue were compared. It is interesting to note that, from the morphological point of view, the endothelium of large veins remained unchanged, endothelial cells preserved their normal appearance, and were devoid of any signs of pathological change, as far as this could be ascertained in a histological preparation. At the same time Kupffer cells absorbed practically no colloid. Only more or less random Ag aggregates can be found in different areas of capillary endothelium. Deposition of Ag in histological preparations stains a light brown. The decrease of adsorption properties of venous and capillary endothelium 24 hr after irradiation, as well as phagocytosis by Kupffer cells—the morphology of these structures being unchanged—can be taken as convincing evidence of alterations of their physical chemical state. It can be assumed that the observed changes of tissue adsorption were caused by a depolymerization of muco- and glucoprotein macromolecules, which reached a maximum 24 hr after irradiation. A disturbance of the normal relationship between macromolecular and highly dispersed fractions in cytoplasm, together with other biological processes, causes changes in surface-active properties of cells. These properties form the basis of adsorption, and of a special example of adsorption—phagocytosis.

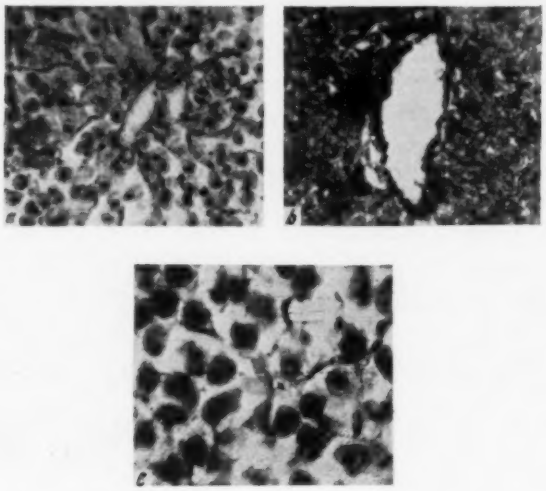

FIG. 1

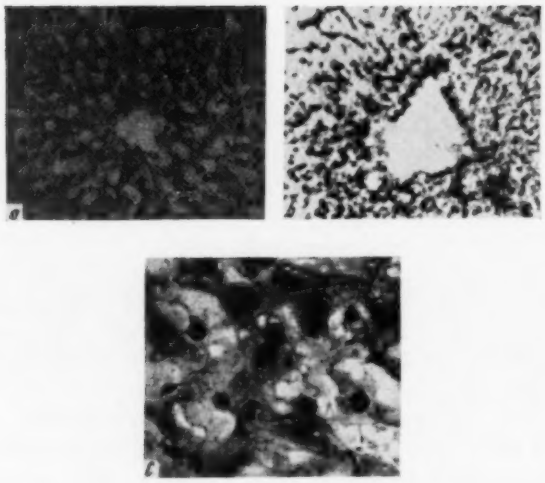

FIG. 2

FIGS. 1–4. Microphotographs of histological preparations of liver after perfusion with colloidal Ag. Normal: (1a). Following irradiation: after 2 hr (2a); after 24 hr (3a); after 48 hr (4a) (×250). Radioautographs (1b, 2b, 3b, 4b) of the same preparations; microphotographs (1c, 2c, 3c, 4c) as above (×60, diminished 4/5).

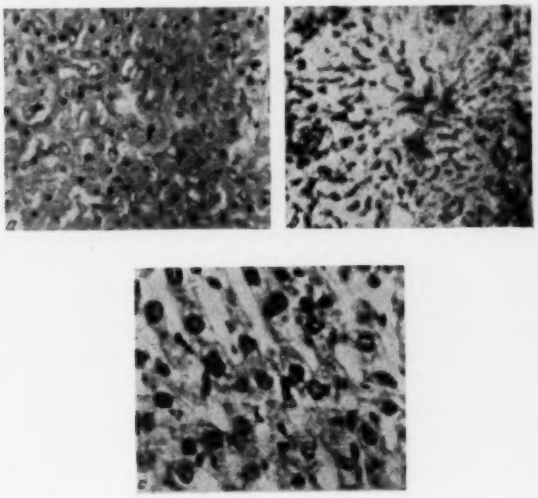

FIG. 3

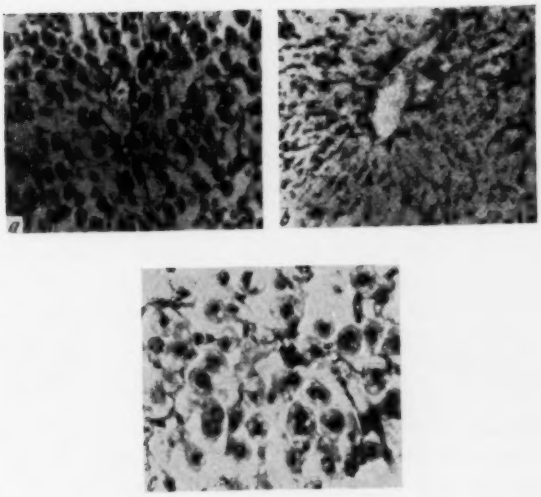

FIG. 4

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Preparations obtained 48 hr after irradiation (Figs. 4*a*, *b* and *c*) exhibited a tendency towards normalization, and even towards an increase in tissue absorption. At this time a marked deposition of colloid in capillary walls could again be observed, along with aggregates in Kupffer cells and capillaries; in histological preparations they were sharply delineated and had a black colour. Whether we are here dealing with a simple restoration of the normal state, or with an increase of absorption capacity due to formation of polymers from products of previous depolymerization (still unpolymerized at 24 hr) must be shown by further investigation of those fine physico-chemical changes in tissue microstructures which have previously escaped our attention.

The preferential aggregation of colloids in liver reticulo-endothelial cells has been long recognized [7]. Application of colloidal Au (radioactive), chrome phosphate and uranium [8] has recently confirmed the distinct differences in intensity of absorption by reticulo-endothelial elements as compared with the surrounding parenchyma. The mechanism of phagocytosis is essentially governed by the same physico-chemical surface forces as other types of adsorption, with an apparent difference in quantitative parameters. It has been shown [9] that the more intensive leucocyte phagocytosis of coal particles than of quartz is primarily caused by the different capacity of coal and quartz to adsorb serum proteins on their surface, and secondly by a different degree of wetability of adsorbed particles by the cytoplasm of phagocytes. A disturbance of the relationships between high polymers and finely dispersed substances in cells under the influence of X-irradiation probably alters the last named of these conditions. It must also be borne in mind that ionic equilibria in tissues can be shifted, since it is known [10] that K^+ inhibits phagocytosis by leucocytes. The details of these problems will be elucidated in further communications.

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SUMMARY

(1) Autoradiographs and histological preparations were prepared from liver perfused with colloidal ^{110}Ag at different periods after whole-body irradiation with 1000 r (X-rays, rats).

(2) It was found that in normal liver tissue, colloidal Ag is markedly phagocytized by Kupffer cells, and adsorbed on the walls of large and small veins and capillaries.

(3) It has been shown that 2 hr after irradiation a decrease in colloidal Ag deposition on the endothelial tissues of vessels occurs; after 24 hr the vascular endothelium adsorbs no colloid; similarly there is a sharp decrease of Ag phagocytosis by Kupffer cells.

(4) The decrease of absorption capacity of liver cell structures for colloidal Ag does not appear to be associated with an alteration in micromorphology, but rather with changes in physico-chemical properties of high polymer compounds normally responsible for colloidal adsorption. It is assumed that the decrease in absorption capacity of tissues is closely associated with a process of depolymerization of high polymer substances in cells, proceeding in a parallel fashion after irradiation.

(5) After 48 hr an increase in tissue adsorption capacity is found, apparently due to polymerization processes. Whether the newly-formed polymers are identical with those of normal tissue will be shown by further investigation.

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THE EFFECT OF X-IRRADIATION OF THE MATERNAL ORGANISM ON FOETAL HISTO-HAEMATIC BARRIERS (MEMBRANE PERMEABILITY)*

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THE effect of X-rays on the embryonic development of animals has been studied by a number of workers. Most reports claim that irradiation of the maternal organism causes foetal death, or the birth of abnormal young.

Chaikovskaya [1] found in the dog that irradiation on the twenty-eighth day of pregnancy with 500 r produced variations of development within the same litter. The weight of the puppies varied up to the seventh month, only one puppy developing normally. With all puppies, there were symptoms of rickets and cardiovascular abnormalities. Irradiation of mice [2] has shown that anomalies and malformations occur in the progeny most often on the ninth and tenth day after conception. In rabbits, the greatest radiosensitivity was observed between the eighth and twelfth days of pregnancy. Some of the foetuses examined at the end of the third week and at the end of pregnancy were found to be macerated with an absence of amniotic fluid and with placentae located in utero.

Irradiation of chicken eggs at early stages of development [4] showed sharp decomposition phenomena within 12 hr after 0·5 of an erythema dose. Recovery took place within 72 hr after irradiation, but various malformations appeared later.

After irradiation of pregnant mice with a dose between 240 and 740 r, it was found [5] that marked changes in the foetal central nervous system (c.n.s.) occur: with 10-day embryos, there was considerable disintegration of nerve cells of brain and spinal cord; with 14-day embryos, sensitivity to irradiation was less marked. High radiosensitivity of nerve cells is also found in the early postnatal period. Thus, irradiation of the skull of newborn mice with a dose of 40 r disrupted many cells in the cerebral hemispheres [6].

Analogous data were obtained [7] from whole-body irradiation of mice and rats

* Biofizika **3:** 2, 226–232, 1958 [Reprint Order No. BIO 132].

with 35–600 r. On the basis of histological examination, the author concluded that neural tissue of embryos appears to be particularly radiosensitive, and that injury depends on the duration of maternal irradiation.

Of particular importance are the findings from pregnant women exposed to atom-bomb explosions at Hiroshima and Nagasaki. A high newborn and foetal death rate was observed if the mother was within 2000 m of the epicentre and was exposed to the irradiation. Mortality and morbidity reached 60 per cent. With pregnant women uninjured at a distance of 4000–5000 m from the epicentre, newborn morbidity and mortality amounted to 6 and 10 per cent respectively. The length and head circumference of the newborns were considerably less in the first group than in the second [8]. There are also findings with children born to parents exposed to X-irradiation. Many abnormalities in foetal development have been described: microcephaly, microphthalmia, cataracts, hydrocephalus, mongolism, deformation of the extremities, abnormal ossification of the skull and other bones.

Thus, on the basis of experimental and clinical data from the majority of reports, the injurious effect of penetrating radiation on growth and foetal development has been proved.

However, it can be seen from the literature that very little attention has been paid to the problem of changes in the foetal histo-haematic barriers due to X-irradiation of pregnant animals. Individual reports deal with the problem of the development of the "blood–brain barrier" in various animals in the course of ontogenesis. Shtern [9] showed that puppies, kittens and young rabbits born with an under-developed brain have a not fully-developed blood–brain barrier. Along with investigation of the blood–brain barrier in newborns, the function of this organ was investigated in the foetus. It was shown that in dog, cat and rabbit foetuses the specificity of the blood–brain barrier is considerably less than in newborns.

A study of the blood–brain barrier in newborn and embryonic guinea-pigs has shown that insufficiency is found only in early foetal stages of development. Guinea-pig embryos at a certain stage of development (appearance of hair and freeing of the ear lobes) possess a fully-developed blood–brain barrier. Since newborn guinea-pigs possess mature nerve centres, these facts confirm the close relationship between the degree of maturity of the c.n.s. and the state of the blood–brain barrier.

In one of these reports [10], where the development of the blood–brain barrier was studied in rabbit foetuses, ^{32}P was used as an indicator of permeability. It was shown that the content of ^{32}P in adult brain tissue was 25 per cent of its concentration in blood by 24 hr after application. In the foetus under the same conditions, the brain contained 6 times as much ^{32}P. Shortly before the end of pregnancy the foetal brain contained 4 times as much ^{32}P as adult c.n.s. tissue. The brain ^{32}P concentration remained high for the first few post-natal weeks, although it was gradually decreasing during this period. It was confirmed that the permeability of the foetal and newborn blood–brain barrier is higher than in the adult. Analogous findings were published by Hess [11].

These reports proved the close relationship between the function of the blood–brain barrier and the existence of a basic "material" (mucopolysaccharide or mucoprotein) in the c.n.s., filling the intercellular space. Thus it has been shown that the blood–brain barrier develops in proportion to overall foetal development.

We have attempted to examine the effect of various doses of X-irradiation of the maternal organism on the development of foetal and newborn barrier functions. Particular attention was devoted to the function of the blood–brain barrier in relation to irradiation of the placental barrier.

Experiments were carried out on rabbits of the chinchilla strain and 202 rabbit foetuses at various stages of development were investigated. Irradiation was carried out with an RUP apparatus (190 kV, 15 mA, 0·75 mm Al + 0·5 mm Cu) with a focal distance of 75 cm, dose rate of 8 r/min, total dose 600 and 1000 r.

Rabbits were irradiated on the twentieth, twenty-fifth, twenty-ninth, and thirtieth day of pregnancy (as is well known, pregnancy lasts 30 days in the rabbit), and ^{32}P as Na_2HPO_4 was used as an indicator of permeability.

Immediately after irradiation the ^{32}P was applied; rabbits were killed by air embolization 2 hr after injection of ^{32}P. The rabbit uterus was opened under local anaesthesia, since maternal anaesthesia considerably effects the state of the foetus. The foetuses were carefully removed, the membranes were removed, and the placenta was separated. In order to prevent foetal blood loss the umbilical cord was tied with silk, and to prevent drying the foetus was immediately placed in a water bath. Simultaneously maternal blood was sampled for counting.

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In the foetus blood, brain, muscle and liver were measured for radioactivity. In the mother, brain, muscle and liver were measured. Tissue samples were taken after previous homogenizing of the part used, or of the total organ, depending on the organ size. The wet samples were weighed and thinly spread on microscope slides with an area density of about 50 mg/2·83 cm^2. The blood sample was taken at the moment of decapitation on to a piece of filter paper, which was then fixed to a glass plate.

Activity of samples after drying was determined with an apparatus of the B type with an Al AC-2 tube. Radioactivity of embryo tissues was expressed in percentage radioactivity of simultaneously sampled embryonic blood, the latter set at 100 per cent; radioactivity of maternal tissues was expressed in relation to the activity of maternal blood. Radioactivity of embryonic blood was determined in relation to maternal blood activity.

It was shown in preliminary experiments that the sixteenth to seventeenth day of pregnancy is the earliest convenient date in studying the formation of foetal barriers. Previous to this period organ size is too small. Experiments carried out after the determination of the indicator dose of ^{32}P injected into the maternal vascular compartment showed that in order to obtain reasonable activity in foetal tissues it is necessary to apply at least 15–20 μc/kg body weight. The time necessary for the penetration of ^{32}P across the placental membrane and then into foetal organs was taken into account.

It was found most convenient to sample material 2 hr after the application of ^{32}P, since before this time tissue activity in the foetus was very low.

First, foetal barrier function was investigated at various stages of development in the normal state without the imposition of further variables.

THE NORMAL STATE OF HISTO-HAEMATIC BARRIERS (MEMBRANE PERMEABILITY) IN THE COURSE OF DEVELOPMENT

Experimental results are shown in Table 1, from which it can be seen that 2 hr after the application of ^{32}P to pregnant rabbits, brain activity in 20-day foetuses

amounted to 58 per cent of foetal blood activity; in 25-day foetuses, 26 per cent; in 29-day foetuses, 18 per cent; and in 30-day foetuses, 17 per cent. The brain content of ^{32}P decreased sharply with development. At the beginning of the third 10-day period of development, brain activity was almost 3·5 times higher than at the end of pregnancy. Parallel investigation of tissues and blood in adult animals (Fig. 1) showed that brain activity 2 hr after application of ^{32}P equalled 12 per cent of a simultaneous blood sample. In comparing data from foetuses and adults, foetal brain activity was shown to be higher at all stages of development than adult tissue, e.g. the brain of 20-day foetuses contained 5 times as much ^{32}P as an adult female rabbit brain; that from a 25-day old, twice as much; that from a 29–30-day old foetuses, 1·5 times as much.

TABLE 1. ^{32}P CONTENT OF EMBRYO TISSUES FROM THE OFFSPRING OF CONTROL AND IRRADIATED MOTHERS 2 HR AFTER APPLICATION OF ^{32}P (EMBRYONIC BLOOD SET AT 100 PER CENT)

Foetal age (days)	Tissue	Control		600 r		1000 r	
		% blood	No. embryos	% blood	No. embryos	% blood	No. embryos
20	Brain	58	9	52	2	36	2
	Muscle	279	2	—	—	130	1
	Liver	900	8	385	2	397	2
25	Brain	26	9	22	5	24	8
	Muscle	243	6	163	2	100	7
	Liver	835	9	477	4	318	7
29	Brain	18	13	15·5	20	18	7
	Muscle	177	10	83	13	100	6
	Liver	540	12	275	18	390	6
30	Brain	17	6	11·5	12	—	—
	Muscle	139	9	63	12	—	—
	Liver	573	9	290	12	—	—

Our data on the development of foetal blood–brain barrier agree with the findings of Bakay [10], who estimated the activity of foetal brain at various developmental stages 24 hr after application of $Na_2H^{32}PO_4$ intravenously to the mother.

Analogous results were obtained in investigating other foetal tissues. Muscle of 20-day foetuses showed a ^{32}P content of 279 per cent as compared with foetal blood, 25-day foetuses 243 per cent, 29-day foetuses 177 per cent and 30-day foetuses 139 per cent. Muscle activity at all stages of foetal development was higher than adult tissue, i.e. in 22–25-day old foetuses by a factor of 4; in 29-day foetuses by a factor of 3; and in 30-day foetuses by a factor of 2·5. This ratio decreased towards the end of pregnancy although the percentage activity of embryonic muscle, as compared with embryonic blood, remained higher than the corresponding adult ratios. Foetal liver appeared to have the highest activity of the tissues measured. Liver from 20-day foetuses was 900 per cent of foetal blood, from 25-day foetuses 835 per cent, from 29 to 30-day foetuses 550 per cent. Towards the end of pregnancy the foetal liver: blood activity ratio is equal to that of adult animals. It therefore follows from this data that under normal developmental conditions the ^{32}P content decreases in all tissues with foetal age.

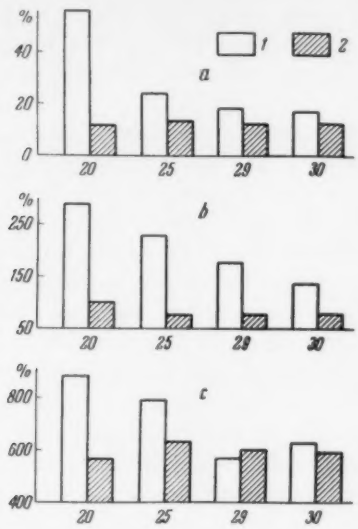

FIG. 1. Radioactivity of maternal and foetal tissues 2 hr after application of ^{32}P (in per cent blood activity). *A:* brain. *B:* muscle. *C:* liver. *1:* foetal tissue. *2:* maternal tissue. Figures below each column represent foetal age in days.

Foetal blood activity, reflecting to some degree the functional state of the placental barrier, was almost always constant and equal to 54 per cent of adult blood activity.

THE EFFECT OF A SINGLE WHOLE-BODY IRRADIATION ON THE STATE OF THE FOETAL BLOOD–BRAIN AND OTHER HISTO-HAEMATIC BARRIERS AT VARIOUS DEVELOPMENTAL STAGES

Table 1 shows the results obtained with a single whole-body irradiation of pregnant rabbits with 600 and 1000 r. On irradiation with 600 r, brain activity of 20-day foetuses decreased by 10 per cent when compared with that of control foetuses of the same foetal age; of 25–29-day foetuses by 15 per cent; of 30-day foetuses by 30 per cent.

Thus a single whole-body dose of 600 r caused a decrease in brain activity at all developmental stages. The same relation was even clearer on irradiation with 1000 r. Under these conditions brain activity of 20-day foetuses amounted to 36 per cent, of 25-day foetuses to 24 per cent and of 29-day foetuses to 18 per cent of simultaneous blood samples, which represented a decrease of 40 per cent with 20-day foetuses and of 8 per cent with 25-day foetuses, as compared with controls. Thus, on irradiation with 600 and 1000 r brain activity decreased at all stages of foetal development. This decrease was enhanced by larger doses.

Analogous relationships were found in a study of other foetal tissues in irradiated animals. Thus, muscle activity of irradiated animals was diminished by 30 per cent with 25-day foetuses, and by 50 per cent with 29–30-day foetuses, as compared with controls.

Muscle activity of rabbit foetuses irradiated with 1000 r was even lower than on irradiation with 600 r. On irradiation with 1000 r it decreased by 54 per cent whit 20-day foetuses, by 60 per cent with 25-day foetuses, and by 44 per cent with 29-day

foetuses, as compared with control muscle activity. A 1000 r dose produced a decrease of foetal liver activity by 50 per cent at all stages of development.

In agreement with the rapid decrease in foetal liver activity, the liver: brain activity ratio was decreased.

Irradiation with 600 and 1000 r at various stages of pregnancy decreased tissue activity as compared with controls. This decrease was less marked in brain, and more marked in muscle and liver in the foetus.

Foetal blood activity of animals receiving 600 r increased with foetal age from 36 per cent (of maternal blood activity) in 20-day foetuses to 67 per cent in 30-day foetuses. An explanation of these findings requires a more detailed comparison of placental barrier permeability with histo-haematic barriers of the foetus.

Penetration of a large amount of ^{32}P into foetal tissues of non-irradiated animals can be explained (as a part of an increased permeability of histo-haematic barriers) by an accumulation of ^{32}P due to absorption and increased incorporation into macromolecular compounds, in relation to an increased intensity of synthetic processes. On irradiation with X-rays of animals at different stages of pregnancy, ^{32}P tissue content in the foetus was always diminished as compared with control tissues. In our experiments a decrease of rabbit foetal tissue activity—after a dose of 600 and 1000 r—was found 60 min after irradiation (Fig. 2).

In studying the penetration of radioactive tracers into tissues from foetal blood, one must take into account the fact that two processes are involved: penetration into

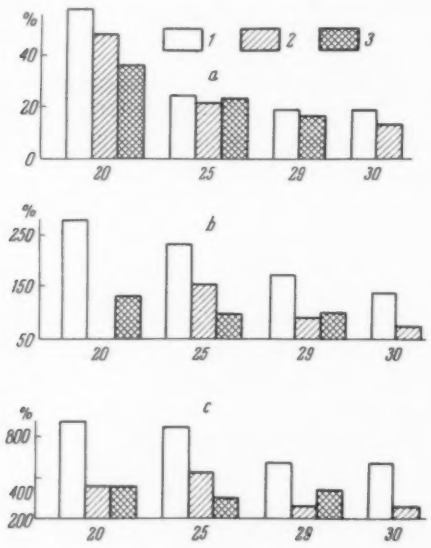

FIG. 2

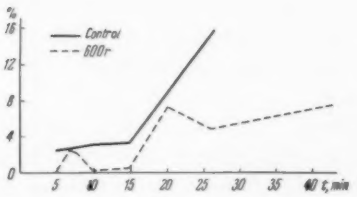

FIG. 3

FIG. 2. The effect of irradiation on ^{32}P content of foetal organs (in per cent of blood activity). *a:* brain. *b:* muscle. *c:* liver. *1:* control. *2:* 600 r. *3:* 1000 r. Figures below each column represent foetal age in days.

FIG. 3. Foetal brain activity at different periods after intraperitoneal application of ^{32}P (in per cent of foetal blood activity).

the tissue, and accumulation in the tissue due to adsorption as well as to incorporation into complex chemical compounds; for this reason we endeavoured to determine foetal tissue activity at different periods after the application of ^{32}P.

A 20-day pregnant rabbit was therefore irradiated with 600 r. The uterine horn was opened 2 hr after irradiation and intraperitoneal ^{32}P was given to each foetus. Tissue activity was estimated at different periods after the application of ^{32}P to the foetus. A control animal was studied at the same time under the same conditions.

Results are presented in Fig. 3. It follows that with foetuses from an irradiated mother, brain activity was low for a period of 15 min, being between 0 and 2·5 per cent as compared with foetal blood. A similar brain activity can be explained by residual blood in the tissue. An increase in brain activity was observed only 20 min after ^{32}P administration. This amounted to 5–7 per cent of foetal blood activity.

Consequently penetration of ^{32}P into foetuses of irradiated animals began 20 min after its application, and by 40 min after application brain activity of the foetus had plateaued. Brain activity of control animals remained constant for the first 10 min after ^{32}P application, whereupon it gradually increased and by 20 min after ^{32}P administration control foetal brain activity was 3 times higher than that of irradiated ones. It follows from these experiments that the transport of ^{32}P in foetuses from irradiated rabbits was delayed in onset and showed a slower time course than in controls.

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CONCLUSIONS

(1) Foetal tissue activity after application of $Na_2H^{32}PO_4$ at all stages of development was always higher than maternal tissue activity.

(2) ^{32}P content of all foetal tissues decreased with foetal age.

(3) On single whole-body irradiation of pregnant rabbits with 600 and 1000 r, foetal tissue activity at all developmental stages was considerably lower than control tissue activity, as measured 2 hr after ^{32}P administration. Tissue activity decreases with irradiation intensity.

(4) Penetration of radioactive substance into the foetus was delayed in irradiated animals and showed a slower time curve than in controls.

Translated by J. CORT

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TECHNIQUES AND APPARATUS

STUDY OF THE RAPID TRANSMIGRATIONS OF SUBSTANCES IN AN ORGANISM USING γ-EMITTING ISOTOPES*

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WHILE artificial radioactive substances are used in biology in a wide variety of ways, attention has been accorded to radioactive tracers mainly for the study of metabolism. The measurement of rates of chemical reactions within the organism, of the nature of intermediary processes, and of the mechanism of chemical transformations, must be seen as the main achievements due to the use of isotopes in biology.

Comparatively little attention has been devoted to questions concerning not so much the elucidation of chemical transformations as the transmigration, in the exact sense, of substances in the organism. This category includes such processes as the circulation of the blood and lymph, absorption, secretion, etc.

Of course, it is not possible to draw a sharp line between these two uses of tracers. The distribution of the substances entering the organism—the phenomena of absorption, secretion, concentration in particular organs and tissues, etc.—is in practice inseparable from the part played by these substances in metabolic processes. In all these cases, however, direct observation of the movement of a tracer and of the dynamics of its distribution throughout the organism has an independent importance of its own.

The blood circulation function occupies a special place in this field of studies. Here registration of the rapid processes by which substances are carried along with the bloodstream is of great interest to research workers, and can become a matter for far-reaching studies. The possibility of observing and assessing very precisely changes in the blood circulation function and in those of absorption, secretion, etc. is of great interest in the study of the regulatory mechanisms of physiological processes, quite apart from the study of the influence on the organism of numerous pharmacological and physical agents. It must be said, however, that while biochemical studies using isotopes have become exceedingly widespread, the physiological aspect has been insufficiently developed.

This situation is not unconnected with the fact that the problems we have labelled "physiological" must be approached technically in ways other than the methods accepted in chemistry and biochemistry for tracing radioactive substances, using a universal registering apparatus.

In the given case, the special feature of the experiment is the use mainly if not

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exclusively of γ-emitting isotopes. Placing the detector outside the organism (near its surface), one must carry on uninterrupted registration of activity in particular sectors. This enables one to gauge the character and the speeds of redistribution of the isotopes introduced into the organism, on the basis of direct measurement *in vivo*, i.e. without making blood tests, tissue biopsy, or extraction and chemical processing of the dead animal's organs. Such an experiment calls for unusual techniques, which in some respects bring with them greater difficulties than the usual preparational and chemical measurements. Having taken a specimen from an organ of a dead animal and measured its activity by means of a counter, one can determine quite unambiguously the radioactivity of that particular piece of tissue; localization of radiation under the conditions of an experiment *in vivo*, measurement being made from a distance, presents certain difficulties. To overcome these difficulties, however, is a task of great importance.

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The technical prospects of development in this field hinge on three points.

(1) A technique is required which will make possible the registration of changes in intensity of radiation at a given point of the organism in time and, furthermore, over a varying range of speeds. One must be able to register processes which take place in the course of a few seconds, when it is a matter of determining the speed of one circulation cycle of the blood, and others which take minutes or tens of minutes, when investigating the dynamics of the accumulation of a substance in certain organs and tissues, or its redistribution.

(2) For a fully valid study of the true nature of the phenomena under observation, it is not sufficient to make measurements at some one point of the organism. A general picture of the processes referred to can be gained only from comparative analysis of phenomena simultaneously in different parts of the organism.

(3) The difficulty lies not so much in preventing the general diffuse γ-radiation of the organism, due to the introduction into it of the radioactive substance, from interfering with the measurement of radiation intensity at one particular chosen spot, as in bringing out even short-lived and very small changes in radiation intensity in time for the given point or by comparison with adjacent sectors. And the intensities to be compared usually vary comparatively little from one another (often by no more than 20–30 per cent).

To bring out such small variations in intensities against the background of the organism's general diffuse radiation, and that dynamically (i.e. when prolonged measurements are impossible because the quantity being measured is changing more or less rapidly), is no easy task. To master it we applied the principle of contrasting variations in intensities by means of the non-linear registration of the γ-radiation. It is this principle which forms the basis of our technique, which is a kind of radiography. This method determines the dynamics of redistribution of a substance within the organism by means of several scintillation counters connected to the apparatus which constitutes a "multi-channel radiograph".

In the first version the UNIR-1* apparatus has two channels [1]. The data given in this paper were obtained using the second model, the UNIR-2, which is a three-channel radiograph. A special feature of this radiograph is the non-linear dependence of its readings on the intensity of the γ-radiation registered. This means that radiation

* UNIR-1: Universal Non-linear Integrating Radiograph, model 1.

of comparatively low intensity is received by the apparatus in reduced form, while radiation of comparatively high intensity (for the given working range of the equipment) evokes exaggeratedly high readings. The radiograph's main working condition is what is called the quadrature response. In this case the output current is proportional to the square of the intensity of γ-radiation registered by the counter. This dependence is expressed thus:

$$i = kI_{\gamma}^{2},$$

i being the radiograph's output current, I_{γ} the intensity of the γ-radiation and k the coefficient of proportionality.

If a point source of γ-radiation changes its position in relation to the counter, the readings of an ordinary apparatus will be in inverse proportion to the square of the distance between the source and the counter. The readings of the radiograph under the same conditions will be in inverse proportion to the fourth power of the distance. Rise and fall in the readings of the radiograph as the radiation source approaches or recedes thus shows up much more sharply than with ordinary apparatus. At the same time, the radiograph has the advantage over a mathematical machine which raises to the square in that with the radiograph statistic fluctuations are not raised to the square, but determined by the ordinary law of distribution. Apart from non-linearity, an important difference between the radiograph and ordinary radiometric apparatus is that the former has additional regulating devices which allow the working regime of each channel to be selected according to the conditions of the experiment and the objects it pursues.

The apparatus has three independent channels and three corresponding scintillation counters* attached to the ends of jointed "arms" which have six degrees of freedom. Readings are recorded by pens writing on a common strip with simultaneous time marking (at intervals of 0·25 sec or 1 sec), with automatic recording of the moment of introduction of the radioactive substance, and with space for notes to be written in by hand (changes in the experimental conditions, for instance).

Independent response regulation for each of the channels is provided for. This includes not only changes in working range corresponding to radiation intensity at the point of installation of each of the counters, but also independent regulation of the amplification coefficient, time constant, and γ-radiation intensity "threshold", beyond which non-linear registration is made. This ability to choose the optimum response for the actual conditions of the experiment, and the further possibility of discriminating against the background created by the γ-radiation of the body as a whole (including that from tracers previously introduced), ensure that small changes in γ-radiation intensity shall show up clearly not only in the first experiment, but also after subsequent introductions of the isotope.

It is essential to stress at this point that with the generally accepted research methods, the presence in an organism of a γ-emitting substance introduced for a first measurement limits prompt repetition of the measurement. This fact is a considerable obstacle to the wider use of γ-emitters in physiological experiments, where repeated observation is often important.

The method of non-linear registration of γ-radiation which we have employed has the advantage over the collinated counter (used with ordinary counting equipment)

* The latest model has four channels and four counters.

in that the non-linear method is more sensitive to small changes in the intensity of γ-radiation and can bring out even short-lived and extremely small variations in radioactivity which are practically speaking not picked up by the collinated counter with linear registration of findings. This advantage can be of decisive importance not only for such things as determining the speed of the bloodstream, as will be shown in detail below, but for a whole series of other *in vivo* investigations.

MODEL EXPERIMENTS

The advantages of non-linear registration of γ-radiation for studies *in vivo* as compared to the usual proportional registration were clearly visible even in the first model experiments. In these experiments the dynamic conditions of various *in vivo* studies were simulated by shifts in the position of the γ-ray sources along the line on which the counters are disposed.

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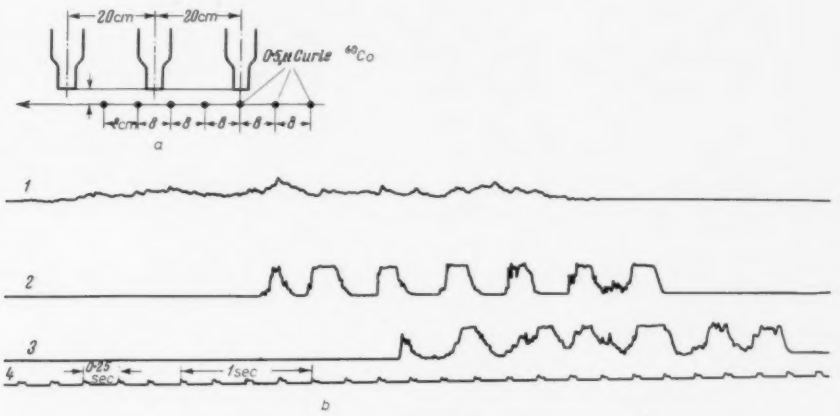

FIG. 1. Diagram of experiment (*a*) and example of record (*b*) with seven specimens each of 0·5μc ^{60}Co moving at a speed of about 20 cm/sec. *1*, for linear response; *2* and *3*, for non-linear; *4*, time marking (0·25 sec).

For comparison, one of the radiograph's channels was switched to give an ordinary linear response, whereas the other two gave their working, non-linear, response. The advantages of the non-linear response came out clearly when a number of equidistant point-sources of γ-radiation were moved rapidly in relation to the counters. Fig. 1(*a*) shows the geometric and dynamic conditions of this model experiment, and Fig. 1(*b*) a sample recording of the results obtained.

As can be seen from Fig. 1(*b*), from a non-linear response (curves *2* and *3*) the moment of the specimens' passage under the counters appeared quite distinctly; the specimens, separated by intervals of 8 cm, could be made out quite well even when moving at a speed of 20 cm/sec. From curve *1*, however, which corresponds to a linear response, it is practically impossible to discover the moment of passage of the different specimens under the counter.

An even clearer demonstration of the advantages of the radiograph is provided when it is necessary to bring out dynamically a small increase in radioactivity above

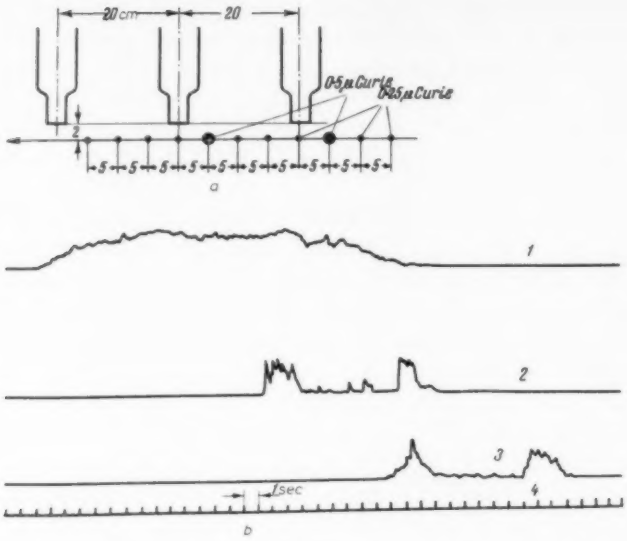

FIG. 2. Diagram of experiment (*a*) and example of record (*b*) with seven specimens of varying radioactivity moving at a speed of 20 cm/sec. *1*, for linear response; *2* and *3*, for non-linear; *4*, time marking (1 sec).

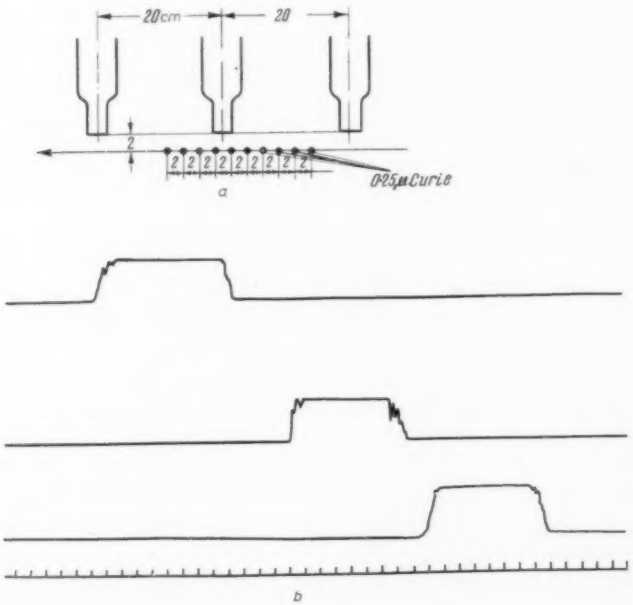

FIG. 3. Diagram of experiment (*a*) and example of record (*b*) with a compact row of ten specimens of 0·25μc ^{60}Co moving at a speed of 2 cm/sec. All three channels gave a non-linear response.

the background. Such a demand sets conditions approaching those prevailing when the second wave in a radiocardiogram must be determined, or a repeat measurement made of the speed of the bloodstream. Model experimental conditions of this kind are reproduced in Fig. 2(*a*), and the results in Fig. 2(*b*). As can be seen from Fig. 2(*b*), from the readings of the linear channel (curve *1*) it is practically impossible to distinguish the moment of passage of a source 0·5μc of ^{60}Co placed among other sources of half this activity. But the two non-linear channels (curves *2* and *3*) show quite distinctly a two-fold increase in radioactivity, this being achieved by appropriate selection of the non-linear response, combined with introduction of discrimination at output.

Another example from the sphere of model experiments shows registration of the front of a radioactive "wave" approaching the counter. In this case the "wave" (resembling, for instance, the movement of a radioactive substance introduced into the blood) is represented by a moving row of 10 small (0·25 μc ^{60}Co) specimens, separated one from another by a distance too small to allow the different specimens in the series to be resolved. Under these conditions the passage under the counter of the whole series is registered, as Fig. 3(*b*) shows, in "table-top" curves (the data of Fig. 3(*b*) are for a non-linear response on all three channels) with exceptionally steep rises and falls in activity.

The examples given of the use of the radiograph in model experiments are evidence that this instrument can be of assistance in dealing with problems that have proved intractable when ordinary equipment was used.

RESEARCH *IN VIVO*

The problem of determining the speed of the bloodstream is one which has long been facing clinicians and physiologists, and which has its own long history of development. Not one of the methods which have been used, however, can be considered as satisfying today's requirements. The difficulty of the task may be illustrated by the fact that even today there are supporters for the use of "sanguinary" methods not only in experiments on animals [2], but in clinical practice, in examinations of patients for diagnostic purposes [3].

That such a situation prevails is not unconnected with the complete unsuitability and practical uselessness of ordinary radiometric apparatus for determining, for instance, the moment of approach to the counter of a radioactive substance within the bloodstream.

The use of a collimator to separate out a narrow directed beam of γ-radiation emanating from a particular part of the organism does not solve the problem fully. The main defect of a collimator used for research into the peripheral circulation is the necessity it involves of introducing comparatively large amounts of the radioactive tracer. No less vital a defect of a collimated counter used with ordinary measuring apparatus is the impossibility of discovering through it small and short-lived changes in the intensity of the radiation registered. In an *in vivo* examination under static conditions, directed towards discovering the metastases of a malignant tumour from the selective absorption of a tracer substance, a collimated scintillation counter will indicate nothing less than a threefold rise in the concentration of the isotope in a small section of tissue as compared with the average concentration in the surrounding

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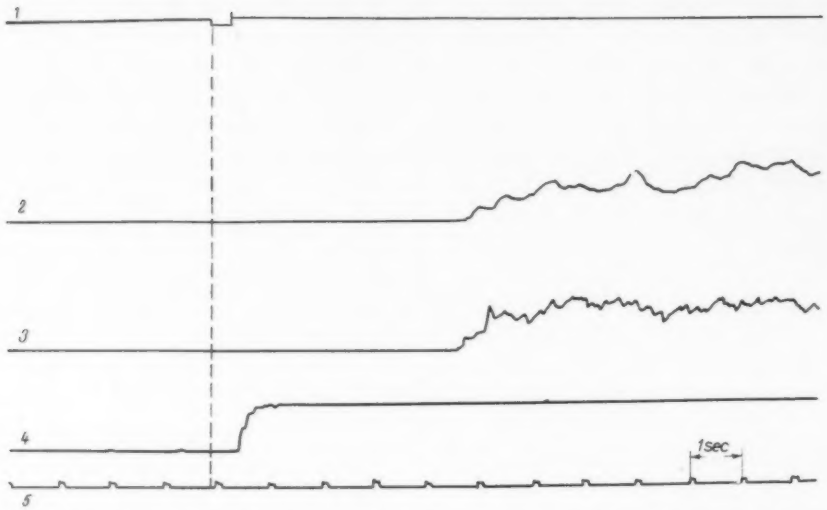

FIG. 4. Example of record in determination of bloodstream speed in a rabbit. *1*, note of moment of introduction of 10μc ^{131}I; *2* and *3*, registration of readings on counters placed symmetrically by right and left femurs; *4*, the same for counter in region of heart; *5*, time marking (sec).

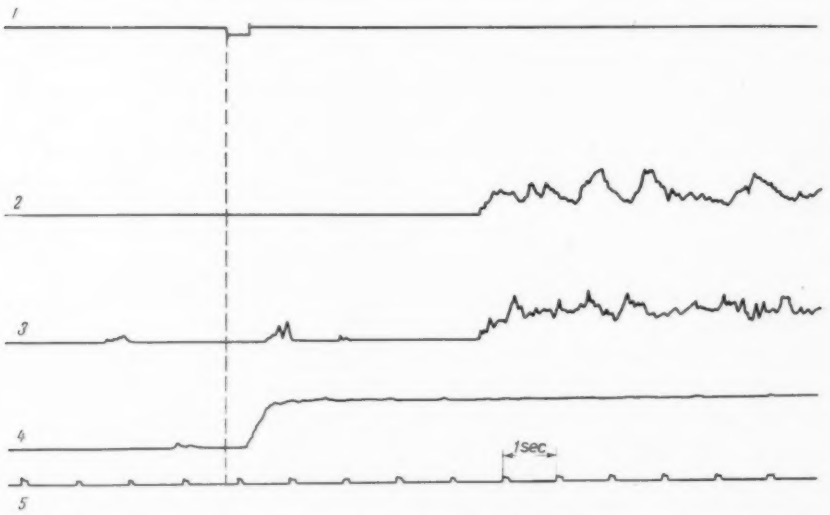

FIG. 5. Example of record in repeat determination of bloodstream speed in the same rabbit (a few minutes later than the first determination). Dose introduced, placing of counters and meaning of curves all as for Fig. 4.

tissues [4]. And if the examination involves the need to make dynamic measurements, then even a three-fold rise in the concentration of the isotope would be obviously insufficient. Yet such a requirement can quite definitely be met, as we have shown in our model experiments, by means of the non-linear method of registration (see Fig. 2, for instance, where a doubling of activity is shown quite clearly under dynamic conditions).

As an example of dynamic measurements *in vivo*, Fig. 4 shows a specimen record of readings taken during determination of bloodstream speed in a rabbit. The experiment was carried out using the radiograph with a non-linear response for all three channels. Injection was made into a vein of the rabbit's ear of 10 μc ^{131}I,* the moment of introduction being automatically noted (*1*, Fig. 4). Two counters were placed symmetrically in the middle region of the right and left femur (*2* and *3*); the third counter was placed in the region of the heart (*4*).

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As can be seen from Fig. 4, the moment of the radioactive substance's arrival from the ear at the site of the counters is clearly shown in curves *2*, *3* and *4*.

As has already been noted, the radiograph provides for regulation of the intensity threshold of γ-radiation above which any rise is registered in non-linear fashion by the apparatus. This ensures that subsequent definitions can be made of the bloodstream speed, with repeated introductions of radioactive tracer, against the background of the isotope introduced earlier.

Fig. 5 shows an example of such a repeat definition of the blood speed, with a second injection of 10 μc ^{131}I a few minutes after the introduction of the first (identical) dose. The positioning of the counters and, accordingly, of the curves, was here the same as in the experiment illustrated in Fig. 4. As Fig. 5 shows, the moment of approach of the radioactive "wave" to the area in which the counters are disposed comes out after the repeat injection of the tracer no less clearly than after the first.

The peculiarities of the rabbit's anatomical structure preclude the making of a radiocardiogram with the counter placed over the heart. So in setting the working response of the appropriate channel of the radiograph the aim set was only to bring up as clearly as possible the arrival of the radioactive blood at the heart. This aim is best achieved under conditions enabling registration of a stepped curve to be made, as is demonstrated by Figs. 4 and 5. The presence of so steep a front ensures a high degree of accuracy in registering the moment of the radioactive blood's approach to the site of the counter, which makes possible a quantitative definition of one of the vital parameters for blood circulation—that of time.

In examining human beings it has as yet not proved possible to register simultaneously both a radiocardiogram and the speed of the peripheral bloodstream. Such data could only be obtained separately, from separate examinations. The factors conditioning the unattainability of simultaneous registration of these data in the course of one examination include not only the non-existence (or rarity) of multichannel installations, but also the considerable difficulties presented by the need to register at one and the same time a comparatively compact portion of radioactive blood in the heart and the very much diluted radioactivity brought by the bloodstream to, say, the lower limbs.

* ^{131}I was used in the experiments on animals to determine the speed of the bloodstream, as a longer-lived "substitute" for the usually used ^{24}Na.

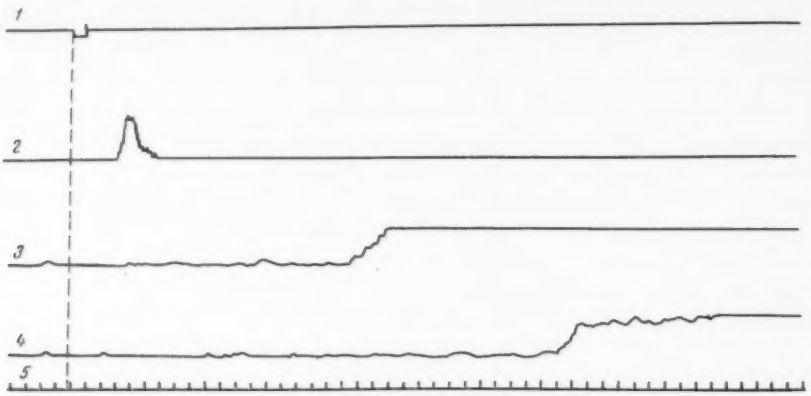

FIG. 6. Radiocardiogram of right side of human heart and determination of bloodstream speed in thigh–shin area. *1*, note of moment of introduction of 50μc ^{24}Na; *2*, regi stration of readings of counter placed by right margin of heart; *3*, the same, for counter by upper third of left femur; *4*, the same, for counter by lower third of left gastrocnemius muscle; *5*, time marking (sec). The sector between the counters by the left leg measures 50 cm, and the interval elapsing during which the blood passes through this sector corresponds to the change in curves *3* and *4* (13 sec).

The possibility of selecting the working response of each of the radiograph's channels independently goes a long way towards solving this problem. Figs. 6 and 7 show the results of examinations made of human beings using radioactive sodium (injection of 50 μc ^{24}Na).

Fig. 6 gives a specimen record made under the following conditions: *1*, automatic recording of the moment of introduction of the 10 μc ^{24}Na into the ulnar vein; *2*, registration of readings with counter placed over the region of the right edge of the heart; sensitivity of the channel is reduced* and "contrast" is high. The narrow peak corresponds to the arrival of the radioactive venous blood from the ulnar vein to the right side of the heart (rising part of curve) and the departure of radioactivity from there into the lungs (falling part of curve). The return of the radioactive blood from the lungs to the left side of the heart is not registered when this response and siting of counter is used. Curves *3* and *4* correspond to the arrival of the blood at the upper part of the femur and at the lower section of the gastrocnemius muscle. The difference in time between the rise of these curves characterizes the time taken by the blood to pass through the sector extending from the hip to the lower third of the shin. The space between the counters was equal, in the case of the person here under examination, to 50 cm, and the time taken by the blood to pass through this sector (judging by the difference between the rise in the curves *3* and *4*, Fig. 6) equal to 13 sec. These data enable the speed of the bloodstream in this given sector to be defined, this being 4 cm/sec.

Fig. 7 gives the results of an examination during which the counter for the heart region was placed over the left ventricle, closer to the median line. The two other

*A dose of 50μc ^{24}Na is employed with the aim of investigating the peripheral circulation, while for taking a radiocardiogram the dose may be several times less; with a dose of 50μc the sensitivity of the channel must be reduced.

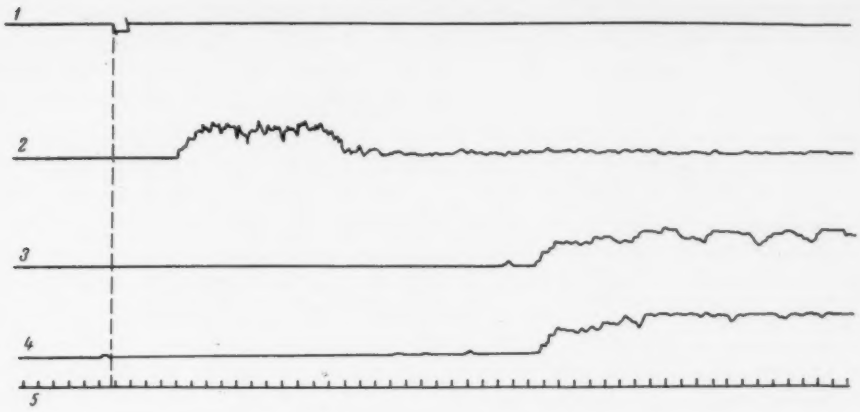

FIG. 7. Radiocardiogram and determination of bloodstream speed in right and left extremities of human being. *1*, note of moment of introduction of 50μc ^{24}Na. Placing of counters: *2*, above heart; *3*, above right gastrocnemius muscle (lower third); *4*, above left gastrocnemius muscle (symmetrically); *5*, time marking (sec).

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counters were placed symmetrically by the lower third of the left and right gastrocnemius muscles (curves *3* and *4*). The simultaneity in the rise of these curves shows the symmetry of the circulation in the lower limbs at this level (lower third of the shin). For the heart region (curve *2*) there is a sharp registration of the moment of arrival of the venous blood to the right side of the heart (rise of curve) and that of departure of the arterial blood from the left side (fall of curve). The "saddle" of the curve, corresponding to the passage of the blood around the lesser circuit, is not sharply expressed, for the contrast definition of the channel was deliberately reduced in connection with the need to register radioactivity in both the right and left sides of the heart. Nevertheless, the overall length of this wide maximum gives an idea of the speed with which the blood passes through the lungs, i.e. of the time taken up in the lesser circuit. It is possible that with a more precise setting of the counter in the region of the left auricle and of the point where the pulmonary artery leaves the right ventricle, to be checked by X-ray examination (as Lammerant and De Visscher [5] did), registration could be made of the "classical" two-peaked radiocardiogram first obtained by Prinzmetal [6]. This was not one of the aims of the present study, however, for the latest model of the four-channel radiograph is more suited to radiocardiography. The small dimensions of the detectors in this latest model enable one to set up one of these in the region of the right ventricle and another, simultaneously, over the left auricle. In this case the first detector should register a curve similar to curve *2* in Fig. 6, and the second one an analogous curve but with a time-lag corresponding to the time taken by the passage of the blood round the lesser circuit. It can thus be supposed possible to produce a two-peak radiocardiogram in the form of two separate peaks, which the above-mentioned authors [5] consider impossible with existing apparatus.

The radiography method may also be employed for study of the speeds of interfusion of substances in the circulating blood, and of their departure from the

blood-vessels, given prolonged registration of radioactivity in the region of the heart. Judging by the data obtained, the interfusion of sodium chloride in the blood is practically over in 40 sec; the subsequent much lower fall in level of radioactivity would seem to be connected with the departure of the sodium from the circulatory system. Some dozens of minutes are required, as we know, for an equal balance to be attained between the sodium in the blood and that in the tissue fluids.

Investigation of the speed of absorption into the blood of substances introduced, say, subcutaneously, can also be made satisfactorily by registering radioactivity in the region of the heart (the second counter may be placed over the point of introduction). After subcutaneous injection of 50 μc ^{24}Na (in the region of the ulnar vein) we observed, starting 15–20 sec after injection, the gradual appearance of and increase in radioactivity of the blood. A level of radioactivity in the region of the heart approximately analogous to that found 30–40 sec after intravenous injection is reached 2–3 min after subcutaneous injection of 50 μc ^{24}Na, although a high level of radioactivity still prevails at this time in the region of the subcutaneous point of introduction.

The speed of absorption thus revealed proved to be considerably higher than had been supposed earlier. This fact is in itself interesting and worthy of further study.

The examples that have been given here do not exhaust the possible uses of the radiograph. It is to be hoped that this method will make possible a real step forward in research involving registration of the transference of γ-emitting isotopes introduced into the organism. The non-linear registration of γ-radiation can also be used in scanning installations intended for the registration of the "distribution map" of an isotope tracer in the organism (radiotopography). In this case, non-linear registration will help to bring out in heightened contrast unevennesses of distribution of the isotope, which will reveal very small increases or decreases in local concentration of the tracer as compared to the surrounding tissues.

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A METHOD FOR STUDYING THE EFFECT OF IONIZING RADIATION ON NERVE CELL FUNCTION*

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MORPHOLOGICAL study of change in the nervous system under the action of radiation has shown the comparatively high resistance of this system compared with other tissues [1, 2]. Some authors, however [3, 4], consider that functional changes in the nervous system set in on the administration of much smaller doses. Functional changes are usually assessed on the basis of data on shifts in e.e.g. and in reflex activity [5, 6]. Direct study of change in the functioning of separate elements of the nervous system, under the action of ionizing radiation, appears justified.

The functions of individual elements in the nervous system seem to be divisible into three classes:

(1) elaboration of signals, i.e. codes (sensitive endings);

(2) transmission of signals (nerve fibres);

(3) translation of signals (synapses and nerve cells).

Most study has been devoted to the action of radiation on the transmission of signals. The majority of authors reach the conclusion that radiation takes effect only upon the administration of large doses, of the order of thousands of rads [7, 8].

It is interesting to elucidate the action of radiation on the elaboration and translation of signals, a problem that has been little studied. So far as we know there has been no research devoted directly to it. A convenient and comparatively simple method for direct study of the elaboration and translation of signals by individual nerve cells is the use of extra-cellular microelectrodes.

The technique we used for preparing liquid extracellular electrodes differs little from the method worked out by Byzov [9]. An electrode of the shape required is drawn by hand out of a thick-walled glass tube, with internal diameter 2 mm, external 3 mm. The tip of the drawn capillary (diameter 50–200μ) is sealed, then sharpened off until an aperture of the required diameter appears. The electrode is filled with a 10% aqueous solution of NaCl.

The signal from the microelectrode is fed by means of platinum wire to the intake of a DB-49 apparatus, which can amplify, record and count the number of nerve impulses from the single cell.† Using this equipment one can study the elaboration and translation of signals in response to specific or non-specific stimuli, and compare these processes in the nerve cells of irradiated and unirradiated animals, and in nerve

* Biofizika **3:** 2, 241–243, 1958 [Reprint Order No. BIO 134].

† The DB-49 apparatus was used for studying the nervous system as early as 1954, by Ardashnikov and Shchipatieva.

tissue preparations. Such a method is not direct, however, since general irradiation of an animal or a preparation may damage not only the cells themselves, but the connections between them, their supply system.

For direct study of the action of radiation on the functioning of the nervous system we have elaborated a method of irradiation through microelectrodes. A source of α- or β-radiation is placed on the tip of a very fine movable needle and inserted into an electrode with an aperture of from 20 to 2μ (see Fig. 1). To begin with the needle is withdrawn from the aperture of the electrode by a distance equal to at least the range of the ionizing particles. The electrode is brought up to the nerve cell, and observations made of the elaboration of nervous impulses as produced normally in response to a specific stimulus (light, extension, etc.). The radiation source is then brought closer to the aperture, and irradiation carried out of the precise cell from

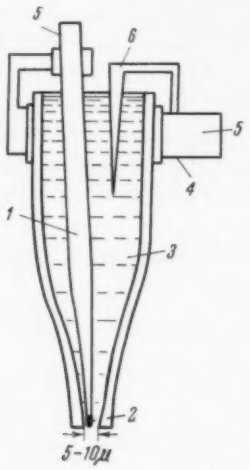

FIG. 1. Microelectrode with incorporated source of radiation. *1*, glass rod; *2*, isotope of ^{32}P, ^{35}S or ^{210}Po; *3*, NaCl solution in agar-agar; *4*, metal fixture attaching electrode to micromanipulator [*5*]; *6*, platinum wire.

which nervous impulses are being tapped. Electrical contact is not broken meanwhile, and the electrical activity of the cell during irradiation can be studied. Irradiation can be interrupted likewise without breaking electrical contact. The construction of this extra-cellular electrode with radiation source is shown in Fig. 1. A β-emitting substance is evaporated on the fine (5–10μ) end of a glass or quartz thread, or inside a fine-walled capillary 10–20μ in diameter; the isotope is covered on top with a film of lacquer 5–10μ thick. An α-emitting substance (e.g. ^{210}Po) can be precipitated electrically on to the tip of the platinum needle, and covered with a 2–5μ thick layer of lacquer. The radiation source is fixed inside the electrode in a particular position, using a micromanipulator. Dosimetry is carried out after the source has been in operation a few times, in order to make sure that the dose rate remains constant.

Let us calculate the activity that needs to be concentrated at the tip of the source in order to produce absorption of a 10 rad/min dose in the cells. Let the diameter of

the cell equal 20–30μ; volume $\simeq 10^4\mu^3 \simeq 10^{-8}$ cm³; and, consequently, the mass of the cell $\simeq 10^{-8}$ g.

One rad = 100 ergs/g = $0{\cdot}6 \times 10^{-8}$ MeV/g = 0·6 MeV/10^{-8} g; i.e. in 1 min 6 MeV of energy of the ionizing radiation should be transmitted to the cell. Passage through the cell of one β-particle of ^{35}S or ^{14}C transmits to it about 50–60 keV on the average, which corresponds to a dose of 0·1 rad; an α-particle transmits at least 3 MeV to the cell, i.e. a "hit" by one α-particle corresponds to an absorbed dose of 2–5 rad. So in order to produce a dose rate of 10 rad/min with an electrode of aperture diameter 10μ ($100\mu^2 = 10^{-6}$ cm²), the aperture must emit 100 β-particles or 2–5 α-particles, and the radiation source itself 10–20 times that number of particles; consequently, the specific activity of the β-radiation source must be equal approximately to 1 mc/cm², and that of an α-radiation source 0·1 mc/cm². It would seem possible to obtain without difficulty a specific activity 100 times as great as this, i.e. to bring the dose rate up to 1000 rad/min.

Calculation gives only the approximate dose. Exact dosimetry can be carried out using counters, a diffusion chamber or a thick-layer emulsion, or by counting the number of particles and measuring the length and the position of the tracks.

The method described can in our opinion also be used for studying the internal structure of the nerve cell. For this purpose different parts of the cell can be damaged by radiation, and the effect studied which this damage has upon the cell's functioning.

Kuzin suggested using the electrode irradiation method for studying change in the polarization and other constants, and in slowly-changing potentials, of cells in different tissues. Employing the technique described, we irradiated with β-particles the ganglionic cells of the retina of an isolated frog's eye. The retina is a convenient model for study of cell activity in the nervous system. Preliminary data show that comparatively small doses (of the order of 100 rad) affect the functional activity of the cells.

In conclusion the author wishes to express his gratitude to M. N. Vaintsvaig for help in his work, and to A. M. Kuzin and N. N. Livshitz for very valuable discussions.

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A METHOD OF PRODUCING INTRACELLULAR MICROELECTRODES*

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THE use of microelectrodes for the study of bioelectric phenomena in separate cells has been increasing over the last decade. The making of such electrodes by hand is a delicate and tedious business. Several varieties of "microforge" have for this reason been suggested for use in their production. Many laboratories, however cannot hope to acquire or make such "microforges", and this is a serious obstacle to the use of this technique in laboratory practice. What is required is a device that can be made by any research worker using the resources ready to hand. The "microforges" proposed by Alexander and Nastuk [1], Bennet *et al.* [2], and Fonbryun's exceedingly complicated apparatus [3], do not meet these requirements, while the production of microelectrodes by the Ottoson and Svaetichin method [4], using a micromanipulator, requires great skill and practice and is unproductive.

The present paper describes a comparatively simple method of making such electrodes. The method is in essence a repeated drawing-out of an ever-narrowing capillary, till this is broken off upon the required diameter being reached. The sections of glass to be made into electrodes are drawn from glass tubing of arbitrary diameter, which is first cleaned with potassium bichromate and then thoroughly washed. We used pyrex tubes of 7 mm diameter, and wall thickness 0·7 mm. The glass used should have a high fusion temperature. Microelectrodes of molybdenum glass proved of little use in practice for intracellular quantitative determinations of potential differences, since when they are filled with KCl solutions there is a deposition of non-soluble crystals, apparently of molybdenum pentachloride.

Firstly, the glass sections are drawn out on a glass-blowing burner, in two stages. First narrow "necks" are made every 3–4 cm along the tube, and the sections of glass thus formed, with drawn-out narrow points at either end, are cut across. Then these narrow points are drawn out again, into a capillary with an external diameter of 0·7–0·3 mm and 5–7 cm in length. The transition from the thick to the thin part should be as sharp as possible (over 3–4 mm). The second drawing gives a considerable reduction in the thickness of the walls of the capillary. The semi-finished electrodes should be preserved from dust. Fig. 1 gives the general principle of the apparatus used for the finishing process. The thick end of the glass tube is fixed in a holder swung from the long arm of a lever, and the other end, the one drawn out into a capillary, is passed through heaters 3, 2 and 1, which are placed on one axis, one vertically below the other.

* Biofizika **3**: 2, 243–245, 1958 [Reprint Order No. BIO 135].

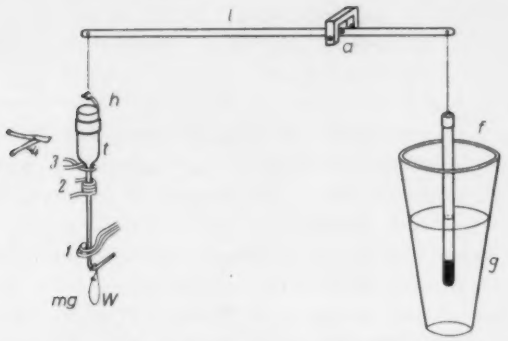

FIG. 1. Diagram of apparatus for making intracellular microelectrodes. *1*, *2*, *3* and *4*, heaters; *t*, glass tube for electrode; *h*, holder; *l*, lever; *a*, axis of lever; *f*, float; *g*, glass of water; *w*, weight; other details not shown.

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The first heater in the form of a hook, is fixed in one position, and is made of nichrome wire of 0·7 mm diameter. The second heater is made of similar wire, and has the form of a quadruple spiral whose internal diameter is 2 mm. The third heater is a loop of 0·2 mm nichrome wire, the diameter of the loop being 1·5 mm. The second and the third heater are mounted on one block, as close as possible to one another. They can be moved about simultaneously in three planes.

Current is supplied to the heaters from an autotransformer (LATR-1) via four separate keys and rheostats. Each heater can thus be switched on and brought up to the heat needed independently of the others. The heat obtained is determined empirically [3]. The capillary is finished off by bringing the first heater up to gentle heat, but not so hot as to allow the capillary to fuse on to the wire, and the end of the capillary on which it is acting is bent into a hook. On the glass hook is hung a small weight, essential for the later drawing-out of the finest tip of the electrode.

From the short arm of the lever is hung a float. This is a glass tube, sealed at one end, and of size and weight such that when plunged into water for half its length it will counterbalance the glass swung in its holder from the other (long) arm of the lever. The float is plunged into a glass of water. When the glass is lowered the unfinished electrode rises until the hook on the end of its capillary catches on heater *1*, which prevents it going up any higher.

All succeeding manipulations are carried out under a binocular stereoscopic microscope (MBS-1). When the second or spiral heater is turned on the capillary starts to be drawn out and up. The degree of tension is determined by how far the glass of water is lowered, or rather by the depth to which the float is under water. The spiral heater brings the thickness of the glass down to 50–70μ. This stage of the process is observed under a magnification to 4·8–8 times. The third heater is then brought up to the "neck" thus formed in the capillary, and its diameter reduced to 8–15μ, this being watched under magnification of 32 times. The shorter the "neck" is, the shorter the fine part of the electrode will be. The heaters therefore must be moved constantly to keep pace with the rising glass. If the glass of water is lifted now, the tension is removed and the glass hook with its weight will hang free.

A fourth heater is now brought up to the very narrowest part of the "neck",

while it is magnified 56 times for observation. The fourth heater is made of platinum wire bent at an angle. The external edge of the angle is flattened, and its thickness must not exceed a few μ [3]. This heater must be brought very close to the site of the final drawing-out, but must not touch the glass. Here it is most convenient to employ the principle of the simple manipulator proposed by Goldacre [5]. This consists of glass slides stuck together with grease and sliding over one another.

The fourth heater is brought up to the temperature required, and the drawing-out process from now on proceeds through the action of the weight. The electrode does not shift in relation to the heater. Heating a well-defined point ensures a sufficiently sharp transition from the comparatively thick section (8–15μ) to the fine tip. The lower section of glass ending in the hook breaks off when the capillary reaches a certain diameter, determined by the size of the weight, and the electrode, freed from the weight, is brought sharply up out of the heating zone owing to the float plunging downwards. This abrupt removal of the electrode from the heating zone prevents the former curling up. As a rule the electrode is not sealed [3].

The diameter of the electrode's tip depends not so much upon the weight diameter and thickness of the original piece of glass as upon the size of the weight that is hung upon it. Experience has shown that a weight of 60 mg will draw electrodes 2·1–2·7μ in diameter, one of 40 mg will draw electrodes 1·2–1·8μ in diameter, and one of 20 mg will draw electrodes with diameters of the order of 0·5μ. From 3–7 electrodes can be drawn in 1 hr by this method.

Measurement of the diameter of the finished electrodes and of the length of their tips can be made only with the aid of a screw ocular micrometer and 450-fold magnification under a microscope. Accuracy will then be ±0·3μ. More exact measurement can be made using an electron microscope. For practical purposes, a more important criterion of the electrodes' usefulness is given by their resistances (see below). It has proved possible to prepare even finer electrodes by means of the device described. Intracellular electrodes are in principle no different from the micropipettes used in micrurgy.

The literature quotes several methods of filling micro-electrodes with the electrolyte. One practice is boiling *in vacuo* for several hours in bidistillate or methylated spirit, the liquid filling the electrode being subsequently replaced by the electrolyte. With this method, however, a considerable number of the electrodes prove useless owing to air-bubbles which cannot be got out of the narrow capillary. We followed the filling method recommended by Caldwell and Downing [6].

The tip of the newly-drawn electrode is plunged into an electrolyte solution for 30 min. The liquid rises in the narrow part of the capillary to a certain level by capillary action. Then the rise of liquid up into the broad end of the capillary slows down sharply. In order to speed up the filling, distilled water is introduced by the broad end of the electrode. In the resultant air-bubble there takes place an exchange of vapours whose saturation pressure is greater upon the pure solvent than upon the solution. The water vapours are precipitated on the column of solution, and in the course of 24–72 hr the bubble rises into the broader portion of the capillary. The water is then replaced by electrolyte without any bubble forming, after which the electrodes are plunged into the solution to obtain an even concentration. The electrodes can be kept in this state for a prolonged period, in a hermetically sealed vessel.

Intracellular electrodes have to satisfy the following requirements: the tip must be exceedingly fine (electrodes with a diameter of 0·5μ are those most commonly used); the transition from the broader section of the capillary (8–15μ) to the finest tip of the electrode (0·5μ) must be by a conical section not more than 200–300μ in length. When the transitional section slopes more gradually the electrodes bend, and when it does so more abruptly they break. But the smaller the internal diameter of the electrode's channel, and the greater its length, the greater also is its resistance. And high resistance in the electrodes complicates greatly the selection of suitable amplifying apparatus. In order to reduce the resistance of the electrodes the conductivity of the electrolyte is often increased. The practice is to fill the electrodes with a 3 M solution of potassium chloride.

Electrodes filled with such a solution and having an external diameter of 2·1–3μ had a resistance of the order of 3–7 MΩ, with a diameter of 1·2–1·8μ their resistance was as high as 15–20 MΩ, and with a diameter of 0·3–0·9μ it was 40 MΩ. When the electrodes were filled with a 0·118 M potassium chloride solution, resistance was of the order of 100 MΩ for an external diameter of about 3μ, 150 MΩ for a diameter of 1·5μ, and 200 MΩ or more for an external diameter of 0·3–0·9μ.

The resistance of the electrodes was determined by the fall in voltage across the electrode as compared to the e.m.f. of the supply. The electrode resistance was most accurately measured when it was nearly equal to the resistance of the amplifier input In order to create optimum conditions during measurement, the amplifier input was shunted with additional resistances.

Microelectrodes made by the method described above show characteristics in no way inferior to electrodes drawn on complex "micro-forges".

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A POTASSIUM STANDARD FOR RADIOACTIVITY MEASUREMENT*

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THE literature sheds insufficient light on methods of measurement of specimens of weak and, especially, natural radioactivity. As a rule, when the radioactivity of specimens is measured, the activity is expressed as the number of disintegrations per unit of time. This mode of expression is, however, conventional and useful only for comparison of different specimens of high activity, measured on one and the same apparatus at one and the same time, i.e. with a small interval of time between the different measurements.

In our laboratory measurement of specimens of weak radioactivity is made taking into account statistical variations and the absorption of β-radiation by a layer of the specimen according to the atomic weight and the thickness of the layer.

Experience has shown that a specimen of given activity, expressed in curies, gives a different number of counts (i.e. disintegrations) per unit of time on different radiometric installations. This is quite natural, since radiation given off by one and the same specimen can give a different degree of ionization in the counting tube according to the latter's specifications, and the mechanical counter will therefore register a different number of impulses even when the counting tubes are of the same type, e.g. MST-17.

For measuring the specimen's activity in curies, we used an active standard made of potassium chloride. The standard specimen must satisfy the following requirements. The radioactive element included in the standard must have a long half-life, be only slightly hygroscopic, be widely found in nature, have one form of radiation with a comparatively stable radiation energy, and, most important of all, it must be easily reproducible in case of replacement being needed.

Natural potassium consists of a mixture of isotopes among which ^{40}K is distinguished by natural radioactivity, with a half-life of $1{\cdot}31 \times 10^9$ years, the radioactive potassium content in the isotope mixture being 0·0119 per cent. Isotope ^{40}K emits β-particles with an energy of 1·325 MeV, β-rays making up 88 per cent of the total radiation; γ-radiation accounts for only 12 per cent, and has an energy of 1·459 MeV.

It should be noted that the efficiency of a γ-quanta count (the number of counts per incident quantum) does not exceed 1–2 per cent, and is often a fraction of 1 per cent. This means that even the most efficient counter records only 1–2 impulses per hundred incident quanta, so that in preparing a potassium standard to give 50 counts/min, one can in practice ignore γ-radiation.

* Biofizika **3**: 2, 246–248, 1958 [Reprint Order No. BIO 136].

A specimen of 0·500 g potassium chloride was tested for ability to absorb moisture from the atmosphere. The experiment showed that this specimen, placed in an atmosphere completely saturated with water vapour, in 12 hr changed its mass through absorption of moisture by 0·121 g, or 24 per cent. Measurement of the specimen's radioactivity showed that this, expressed in counts/min, had changed by only 3 counts (time during which measurement was made: 200 min) as compared with the same specimen in an absolutely dry state, when it gave 45 counts/min.

The same specimen, previously dried out in a thermostat, was measured for radioactivity on an apparatus of type *B* and then, after being left for a prolonged period in an atmosphere with a relative humidity of 78 per cent, was again tested for radioactivity. Repeated measurements of the absolutely dry specimen and the same specimen after remaining in an atmosphere with the indicated humidity all gave the same results within the limits of error.

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Potassium chloride then, as compared to other potassium compounds (K_2Co_3; $K_2Cr_2O_7$; KBr), has a very low atmospheric moisture absorbency, and consequently does not change its radioactivity when the relative humidity varies, an important point in choosing a radioactive substance as a standard. These physical and chemical properties make potassium chloride the most suitable radioactive substance from which to make a conventional standard in relation to which comparative measurements of specimens of low radioactivity can be made. Such specimens must often be dealt with in the practice of a laboratory for biochemical research into radioactivity.

Out of all the methods we have tried for making the potassium standard, the following seems to us the most suitable for practical purposes. Potassium chloride powder of varying mass is placed in cardboard ampoules of cylindrical shape, diameter 15 mm (the size of the window of an end-type counter) and length 1–2 mm; the powder is distributed evenly over the bottom of the ampoule, after which the latter is sealed with fine cigarette paper.

The table shows the results of measurement of the radioactivity of potassium chloride specimens in counts and in curies, according to the thickness of the layer of substance in the specimen. The maximum recorded activity in a potassium chloride specimen, marked N_k in Table 1, was obtained by us by experiment and equals 48·5 counts/min.

The β-particle absorption coefficients in strata of varying thickness can be determined from the graph reproduced in Fig. 1 (column 5 of Table 1).

A potassium chloride specimen with a mass of 108 mg and a thickness of 61·4 mg/cm² was taken by us to serve as a conventional potassium standard with an absorption coefficient of 1·08, determined graphically from the relationship

$$K_c = N/N'.$$

The radioactivity of such a standard of mass M of potassium isotope mixture can be calculated from the well-known formula $I = \lambda N$.

$$N = (6{\cdot}02 \times 10^{23} \times 0{\cdot}0119M)/(40 \times 100),$$

where N is the number of atoms of ^{40}K.

TABLE 1. MEASUREMENT OF RADIATION FROM POTASSIUM CHLORIDE

Mass of KCl (mg)	Thickness of stratum in specimen (mg/cm²)	No. of counts above background in 1 min, N_n	$\frac{N_n}{N_h}$	Beta-part. absorption coefficient in stratum of specimen	Radioactivity (10^{-12}c)	
					Calculated	Measured
50	28·3	6·8	0·14	1·0	23	23
100	56·3	13·0	0·268	1·06	46	46·5
108	61·4	13·7	0·282	1·08	50	50
150	84·9	18·8	0·388	1·09	69	69·5
200	113·2	23·4	0·482	1·17	92	92·5
250	142·0	28·5	0·590	1·21	115	116·0
300	169·8	32·7	0·675	1·26	138	138·0
350	198·1	35·6	0·740	1·34	161	161·0
400	227·2	39·8	0·820	1·37	184	184·0
450	254·7	42·7	0·885	1·43	207	206·0
500	284·0	44·9	0·925	1·53	230	232·0
600	339·6	46·1	0·970	1·75	276	271·0
700	396·8	48·0	0·990	—	322	—
800	454·0	48·5	1·00	—	368	—

Since ^{40}K emits 88 β-particles per 100 disintegrations, and the constant of disintegration

$$\lambda = 0{\cdot}693/T \text{ sec}^{-1},$$

then, substituting all the numerical values, we shall obtain the number of β-particles emitted per second:

$$A_c = \frac{0{\cdot}0119 \times 6{\cdot}02 \times 10^{23} \times 88 \times 0{\cdot}693 \times M}{100 \times 40 \times 100 \times 1{\cdot}31 \times 10^9 \times 365 \times 24 \times 360} = M \times 264{\cdot}4 \text{ particles/sec.}$$

Knowing A_c, the number of impulses of standard N_c to the time unit of the

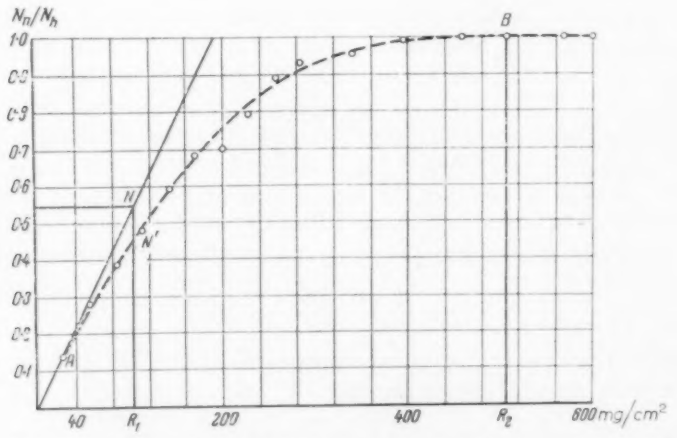

FIG. 1. Absorption of β-particles in a KCl layer of varying thickness. R_1: layer thickness of specimen of KCl mass 108 mg; N^1 and N, points corresponding to measured and absolute radioactivity of the specimen.

counting apparatus, the number of impulses of the specimen N_n, one can determine the radioactivity of the specimen with β-radiation from the relationship:

$$A_n = N_n.A_c.K_n/(N_c.K_c) \text{ particles/sec},$$

where A_c is the number of β-particles emitted by the standard in 1 sec, and K_n and K_c are the absorption coefficients of the specimen and the standard respectively.

In calculating the radioactivity of an unknown specimen, using a potassium standard, one must bear in mind that any specimen, like the chosen standard, is characterized by its own absorption curve, and that in measuring the radioactivity of the specimens the layer should be as thin as possible. For example, the K_c for a mass of potassium chloride of 50 mg is equal to 1. Biological specimens (blood, muscle tissue) with a mass less than 50 mg have a β-particle absorption coefficient, practically speaking, that is also 1, and that can therefore be disregarded.

The method of preparation of the potassium standard which we have proposed is very simple, and within the possibilities of many radiological laboratories. When such a standard is available, measurement can be made of various radioactive specimens with β-radiation, to define their relative and absolute radioactivity. Such a standard in a laboratory provides the means of tracing the change in radioactivity of rapidly disintegrating specimens, also of checking the steady functioning of counting apparatus and the efficiency of a counter, which is very important for measurement of specimens of weak radioactivity and short half-lives.

Translated by R. KISCH

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AN APPARATUS FOR AUTOMATIC PHOTO-RECORDING FROM CINE-FILM OF PUPILLARY REACTION CURVES AND MOVEMENTS OF THE EYE*

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THE reactions of the pupils can be used as a precise and very sensitive indicator of physiological processes only on condition that they are registered objectively. Hitherto, however, in the majority of studies methods were followed which were based on a subjective assessment of the pupillary reflexes (pupilloscopes and pupillometers of various designs). At the same time, attempts have long been in progress to achieve objective registration of pupillary reactions. An original apparatus for dynamic registration of pupillary reactions was proposed by Bellyaminov [1]. In this apparatus the image of the pupil is projected into the plane of a slotted diaphragm in such a way that its horizontal diameter coincides with the slot, behind which is a moving photographic paper receiving the image of the pupil as an unbroken band.

* Biofizika **3:** 2, 248–251, 1958 [Reprint Order No. BIO 137].

Reduction of the diameter of the pupil causes this band to narrow. The slightest vertical movement of the eye, however, means that the diameter of the pupil will cease to coincide with the slot, and on the curve obtained an eye movement may be wrongly interpreted as a pupillary reaction. Thus Bellyaminov's principle admits of a trustworthy result being obtained only if the position of the head and eye is rigidly fixed. In order to attain this immobilization of the eye, the author of the method, who was experimenting with rabbits and cats, had recourse to deep anesthesia, fixed the eyelids by blepharostasis, and cut completely through all the external muscles controlling eye movements.

Glezer [2], Zelondzhev [3] and Liberman [4] have employed the Bellyaminov principle in studies with human subjects. But with a human subject it is impossible to achieve complete immobility of the eyeballs. For this reason application of this principle to study of pupillary reflexes in man does not yield trustworthy results.

Equally searching demands of immobility of the head and eye are imposed by the photo-electric method, which is based on reflexion of a beam of infra-red rays from the iris, to be picked up by a photo-element. The quantity of these rays varies according to the changes in diameter of the pupil.

Matthes [5], Cuppers [6] and Ataiev [7] have used this method for study of pupillary reflexes in man. Recently, however, a number of authors Peterson [8], Loewenfeld [9] have pointed to the imperfect nature of the photo-electric method and the dependence of the curve obtained upon movements of the eye and a number of other, in practice unavoidable, side factors. Special control experiments [9] have established the basic causes of errors in the photo-electric method. These include: variations in pigmentation of different eyes, variations in reflection of infra-red rays from different parts of one and the same eye, and continual head and eye movements by the subject.

As the author indicates, each of these sources of error distorts the measurement by not less than 50 per cent. Furthermore, a curve obtained by the photo-electric method does not convey any idea of the diameter of the pupils and of the amplitude of the reaction in millimetres. Without data on the reaction's amplitude, one cannot calculate its speed. All these shortcomings of the photo-electric method considerably limit its application.

The most precise data on the state of the pupils can be obtained from use of the cinematographic method, which in contradistinction to other methods does not require strict immobility of the head and eyes. But for taking pictures on cinematograph film strong background lighting is needed, creating non-physiological conditions for the study of pupillary reactions (light causing contraction of the pupils).

In the technique we have elaborated this shortcoming of the cinematographic method is avoided, as explained in earlier published works [10]. The registration of the pupillary reflexes is made at a sufficient speed (6–10 frames/sec), with weak background lighting, 15–25 lx at eye level.

Another considerable obstacle to cine-registration of pupillary reactions is the necessity of drawing the curves from the cine-frames. This process is complicated, laborious and a possible source of subjective errors. Lowenstein [11] has attempted to overcome this disadvantage of the cinematographic principle by means of a complex apparatus recently constructed in his laboratory. In this installation the image of

the pupil is projected from the cine-film on to a photo-element. The electric current arising in the photo-element is amplified and recorded by an oscillograph. It is difficult, though, to count upon getting a reliable result from photo-electric recording of pupillary reaction curves from cine-film. Apart from the same disadvantages which apply to photo-electric recording direct from the eye, here there is a whole series of further sources of error. The slightest defect in the film stock, a scratch, a speck of dust, a tiny crystal of fixing agent, or an unevenness in development of the film, must be reflected in the curve. Peterson [8] has a critical attitude to this installation. Lowenstein himself considers that it does not solve the problem of recording pupillary reaction curves from film, but enables one to acquire only a relative impression of the presence or absence of a reaction. In the author's opinion, in order to obtain more exact and reliable results after photo-electric recording of this kind it is still necessary to project the film on to a screen and measure the diameters of the pupils with dividers.

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We [12] constructed an apparatus for measuring the image of the pupils on a cine-film which considerably simplified the process of obtaining the curves, but which still required observation to be made of the image of the pupil on each separate frame, with the setting-up that this calls for. At the present time the whole process of recording pupillary reaction curves from cine-film has been made fully automatic by us, by means of a special apparatus which makes possible measurement of the pupil's image on the film without the direct participation of the research worker.

In this apparatus the image of the pupil from the cine-frame is projected on to the plane of a slot diaphragm *AB* (Fig. 1) and moves at a constant speed in a direction perpendicular to the slot. Behind the slot diaphragm is an unexposed photographic film, on which is recorded, under the given conditions, the greatest diameter of the pupil, in the form of a small column.

A sequence of such copies of the cine-frame gives a curve for changes in diameter of the pupil with time (Figs. 2 and 3). The vertical movements of the eye recorded by the cine-film do not influence the curve. At the same time the horizontal movements

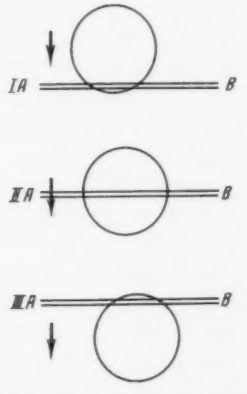

FIG. 1. Diagram showing principle of apparatus for photo-recording of curves. *I*, *II*, and *III*—successive positions of image of pupil in relation to slot diaphragm *AB*, behind which is a film. In position *II* the greatest diameter of the pupil coincides with the slot.

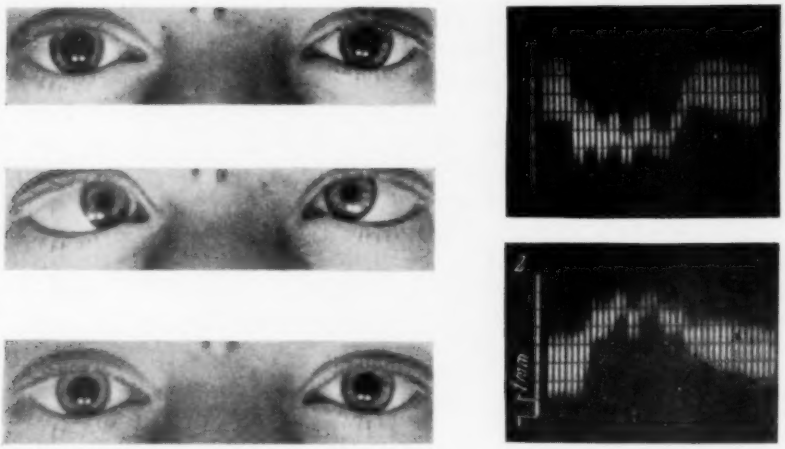

FIG. 2. Reactions of pupils to convergence. Right: curves of pupillary reactions and movements of right (*1*) and left (*2*) eye. Six successive small columns correspond to 1 sec (speed of film—6 frames per sec). At the beginning of each curve there is a scale marking. The space between two divisions on this scale is 2 mm. Left: from top—prints from the 1st, 14th and 37th frames of the cine-film from which the curves are taken.

of the eye lead to a shift of the columns in the curve without affecting their size (Fig. 2). The middle of each column corresponds to the centre of the pupil. From the curve obtained in this fashion one can therefore judge not only of the reactions of the pupil, but also of horizontal eye movements (in nystagmus, convergence, etc.).

Fig. 4 shows a photograph of the apparatus for photographic recording of pupillary reaction and eye movement curves from cine-film, and Fig. 5 illustrates the paths of the rays in this apparatus. A beam of light reflected from a small mirror (Fig. 5*C*) is directed on to a platform with film lying upon it. The image of the pupil on the film *AB* is focussed by the lens *E*. The beam of light then passes through the semi-transparent plate *D*. The lens *F* focuses the enlarged image of the pupil A_1B_1 on to the plane of the exit slot *H*. Close up against the slot *H* is the reel of film *K*. By means of the semi-transparent plate *D* and the eye-piece *L* one can observe visually the image of the pupil on the film (which is vital for focusing purposes).

The slot diaphragm has incorporated in it a transparent film with divisions marked upon it. This scale is printed on every column, which enables one to judge of the absolute size in millimetres of the pupillary diameters. The progress of the cine-film carrying the images of the pupils along its channel (see Fig. 4*b*) is ensured by a synchronous electric motor (see Fig. 4*e*). This turns a special small roller to which contacts are attached. Bringing these together switches on an electromagnetic relay that opens the gate in the film chamber (Fig. 4*a*) and moves the film up. The forward movement of the film by 1 mm corresponds to the forward movement of the cine-film in its channel by one frame. The switching open of the gate takes place for a small space of time during which the image of the pupil on each frame passes

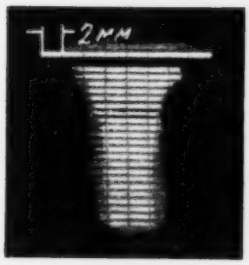

FIG. 3

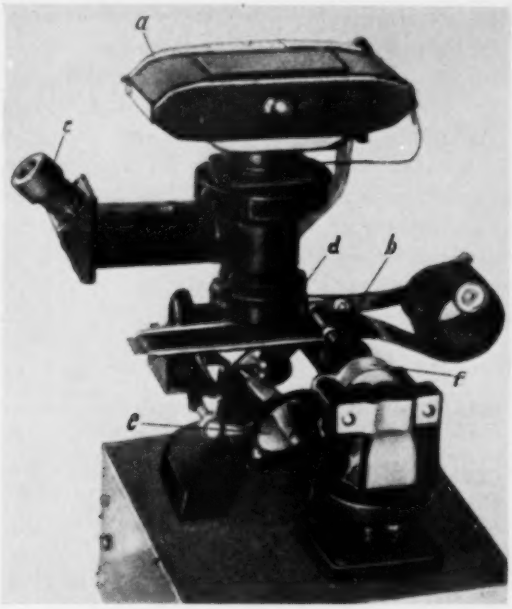

FIG. 4

FIG. 3. Pupillary reflex to light. The light acts out of complete darkness. The recording of the curve starts at the moment of application of the light stimulus. Speed of film, 10 frames per sec. Latent period of reflex to light—0·2 sec (two columns in the curve).
FIG. 4. General view of apparatus. (Letters altered to English equivalents in alphabetical order.) *a*—film chamber; *b*—film channel; *c*—eyepiece; *d*—lens; *e*—mirror; *f*—motor.

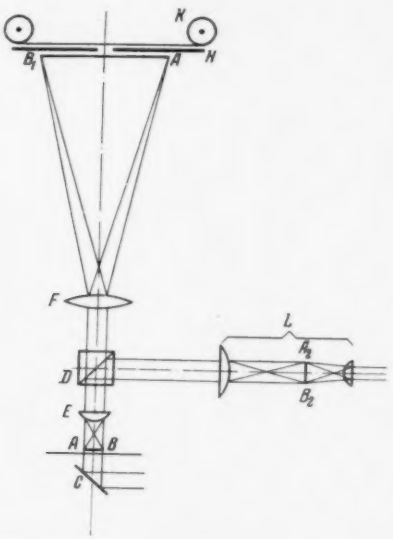

FIG. 5. Progress of rays in photo-recording apparatus. (Explanation given in text.)

by the slot diaphragm its greatest vertical measurement (from upper to lower eyelid). Under these conditions the photographic film which is behind the slot receives the imprint of the greatest horizontal diameter of the pupil in the form of a column. These columns, obtained from the cine-frames in sequence, together form a curve; each column in the curve can equal only the greatest horizontal diameter of the pupil and is the result of the direct projection of that diameter on to light-sensitive materials. The size of each column is not dependent upon eye movements.

The method we have elaborated makes it possible to obtain considerably more precise data on the nature of pupillary reactions and their dependence upon movements of the eyeballs than have been achieved by all the techniques previously employed.

Translated by R. KISCH

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ELECTRON MICROSCOPE STUDY OF THE DISTRIBUTION OF ROD-SHAPED PARTICLES OF TOBACCO MOSAIC VIRUS IN THE VEGETATIVE GEMMA OF THE AFFECTED TOMATO*

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VOL. 3 1958

THE study of tobacco mosaic virus (TMV) in the tissues of the vegatative gemma of the affected plant is particularly interesting, since, as is well known, the first signs of disease appear on the youngest leaves of the infected plant, irrespective of the site of inoculation. It would be natural to suppose that the process of virus reproduction is most rapid in the growing point and the zones adjacent to it. The object of our work was to trace the distribution of the TMV in the vegetative gemmae of tomatoes affected by the disease. We wished to find out whether the rod-shaped particles of the virus occurred in the meristematic tissue and, if they did not, to find out where they could be found and what factors preceded their appearance.

The vegetative gemmae were taken from the side-shoots of old tomato plants growing under cover which had been affected by mosaic for a long time. The growing point and the leaves immediately adjacent to it† were removed, the operation being observed through a MBI-2 binocular microscope. The parts removed were washed with distilled water for an hour, on a filter paper, in order to remove any virus which had reached them from the deeper tissues. This washing was sufficient to remove any virus particles which had been carried on to the surface of the excised parts in sap.

The primary leaves of the tomato vegetative gemmae are, usually, still in the form of meristematic protuberances. The secondary leaves, the dimensions of which do not exceed 1–2 mm, already have tissues in their upper parts which have turned green, indicating the presence of fairly well developed chloroplasts.

Fig. 1 is a sketch of the vegetative growing point, indicating the zones which were studied.

We tested the excised tissues for the presence of virus by three methods: first by testing its infective power by inoculating leaves of *N. glutinosa*; secondly by examining sap from these tissues under the electron microscope, counting the particles; and thirdly by studying the meristematic cells and the cells immediately adjacent to them in ultra-thin sections.

A standardized method of making the preparations ensured that comparable results were obtained.

* Biofizika **3**: 2, 252–256, 1958 [Reprint Order No. BIO 138].

† Hereafter the buds of the topmost leaves will be called primary, and the leaves next to them will be called secondary.

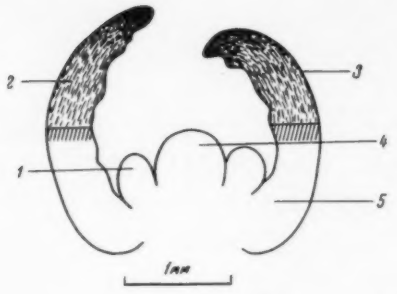

FIG. 1. Diagram of the portion cut from the vegetative gemma of the tomato. *1*—primary leaf; *2*—secondary leaf; *3*—chlorophyll-bearing tissue; *4*—growing point; *5*—meristematic tissue.

In experiment 1 we tested the infective power of a suspension prepared from the meristematic tissue. For this purpose a large number of growing points and the sharp primary leaf buds adjacent to them were excised; the total weight obtained was 2·5 mg. The secondary leaves were collected separately; their total weight was 5 mg. The tissues were ground up in a miniature glass mortar, to which distilled water was gradually added, in the proportion of 1: 200 to the weight of the tissue.

Preparations for examination under the electron microscope were made from the liquids so obtained, and then halves of the leaves of *N. glutinosa* were smeared with these liquids. The other halves were used as a control, being infected with sap from fully-developed leaves diluted to the same extent. After three days the necroses which had formed were counted.

A total of 160 necroses had formed on the 9 control halves of *N. glutinosa*, which had been inoculated with sap from full-grown affected leaves; 27 necroses, i.e. 17 per cent of the control number, formed on the halves which had been inoculated with a suspension made from the meristematic tissue.

In the second part of the experiment 17 control halves of *N. glutinosa* leaves formed 407 necroses, whereas those infected with sap from the second pair of leaves formed 214 necroses, i.e. 52 per cent of the control total.

This experiment showed quite clearly that the amount of virus in the meristematic tissue is considerably less than that in the fully-developed leaves and in the young secondary leaves near the growing point.

In experiment 2 the growing point was cut from the apical bud, but the primary leaves were left; the secondary leaves were cut in half, so as to separate the more developed part of the leaf, which had already turned green, from the basal (younger) part. The growing points and the pieces of secondary leaf were treated as described above, and preparations were made from the suspensions so obtained, as well as from the control liquid, for examination under the electron microscope. It was not possible to obtain enough growing points to infect even 10 leaves; the suspension was only sufficient to infect 5 halves of *N. glutinosa*.

When the necroses were counted, the following results were obtained: three necroses were formed on 5 half leaves of *N. glutinosa* as a result of inoculation with the suspension prepared from the growing points; the control suspension produced

103 necroses. The suspension prepared from the basal part of the secondary leaves produced 17 necroses as against 75 on the control halves. Sap from the green upper parts of these leaves produced 68 necroses on 6 halves, and the control sap, from fully-developed leaves, produced 119 necroses. For the sake of clarity, the results of both experiments and the number of virus particles counted in these tests, which are discussed below, are shown in tabular form.

TABLE 1. THE VIRUS TITRE AND THE NUMBER OF BACILLUS-LIKE PARTICLES OF TMV COUNTED IN VARIOUS TISSUES OF THE AFFECTED TOMATO

No. of expt.	Tissue from which the test material was taken	Number of leaves of *N. glutinosa* used	Total necroses on half leaves *N. glutinosa*	Percentage of necroses	No. of fields of vision	Total number of virus particles
1	Growing point and primary leaves	—	27	17	200 in each field of view	25
	Control (fully grown leaves from same shoot)	9	160	100		~
	Secondary leaves	17	214	52	—	—
	Control	—	407	100	in each field of view	~
2	Growing points	5	3	3	107	8
	Control	—	103	100	in each field of view	~
	Basal part of secondary leaves	5	17	23	100 in each field if view	232 ~
	Control	—	75	100		
	Apical part of secondary leaves	6	68	57	—	—
	Control	—	119	100	—	—

Note. The dilution was 1: 200 by weight.

It is apparent from Table 1 that the amount of virus available in the growing points to produce necroses on *N. glutinosa* was negligible. There was little virus in the basal part of the secondary leaves, but considerably more was found in the upper part of these leaves, where the cells are more specialized. However, the virus titre here was only about half of that in fully-developed leaves from the same shoots.

Examination under the electron microscope revealed a large number of bacillus-like particles in the suspension prepared from fully-developed (control) leaves of affected plants (Fig. 2(1)). Since they were clumped together the separate particles were not counted in this test, the number of particles being conditionally represented by the infinity sign ∞.

Examination of 220 areas in a suspension prepared from the growing points and primary and secondary leaves revealed a total of 25 bacillus-like particles of the tobacco mosaic virus. Apart from these we could not find any bodies with a definite shape, the field of view of the miroscope being dotted with shapeless fragments of damaged tissue. In the second experiment the examination of 107 areas of an extract made from the growing points revealed only 8 virus particles. Fig. 2(2) shows a typical field of view in this test, containing small fragments of tissue and a complete absence of uniform particles.

Sap from the secondary leaves was found to contain a fairly large number of bacillus-like particles and an even greater number of small granules. Separate granules have the appearance of small spherical bodies with a diameter corresponding to that of the transverse section of a virus particle. These bodies may be separated from each other or be joined in groups of two or three. Sometimes they form large groups, consisting of a large number of separate granules. The bacillus-like particles and the granules often form clumps, which makes it impossible to count the virus particles (Fig. 2(3)).

A total of 232 virus particles was found in 100 areas of sap from the basal part of the secondary leaves. The granularity which had been apparent in sap from the secondary leaves in the first experiment could also be seen in this case, but to a lesser extent.

All the areas of sap from the apical parts of the secondary leaves contained a large number of bacillus-like virus particles, which were almost always well shaped. It is possible to see that some of the bacillus-like particles are in fact chains of 7–8 granules (these are indicated by arrows in Fig. 2(4)). Besides the virus particles, this test revealed a large number of small granules scattered more or less evenly over the preparation or forming large clumps. For control purposes we examined an extract from the corresponding tissues of healthy plants; there were no bacillus-like particles.

Suspensions from the growing points of healthy plants, and those from the growing points of affected plants (Fig. 2(5)), contain particles of varying sizes; sometimes these particles are rounded in shape.

Sap from the secondary leaves of the vegetative gemmae of healthy plants contains a considerable number of small particles; the dimensions of some of these correspond to those of particles from the sap of affected plants, but they are more often of irregular shape and "eroded" at the edges. The shadow cast by them after shadowing is less pronounced, which indicates that they are flatter in shape.

Separate granules often occurred in these specimens (Fig. 2(6)); these occur much less frequently in samples from affected plants. A comparison of the results obtained from electron microscopic examination of suspensions prepared from various tissues of the vegetative gemmae of affected plants with the results of testing them for infective qualities shows clearly that they are in close agreement. For example, the virus does not occur in the meristematic tissue of affected tomatoes in its normal bacillus-like form, and the infective power of an extract from this part of the plant is negligible. As the extent of tissue differentiation increases, so the number of particles in these tissues grows and their infective power increases.

The next stage of the work was to study the cells of the vegetative gemma of the tomato in ultra-thin sections. The growing points and the primary and secondary leaves were fixed in 1 per cent buffered osmium tetroxide with a pH value of 7·4 for 2–12 hr; they were then rinsed quickly in water and dehydrated by alcohol. The product so obtained was poured into a mixture of butyl methacrylate (4 parts) and methyl methacrylate (1 part). In order to study viruses it is essential that preparations should be treated with metal. This necessitated working with preparations which had been washed free of methacrylate and shadowed with chromium. The material was examined under a UEM-100 electron microscope.

The most outstanding feature visible on photographs of sections cut from healthy

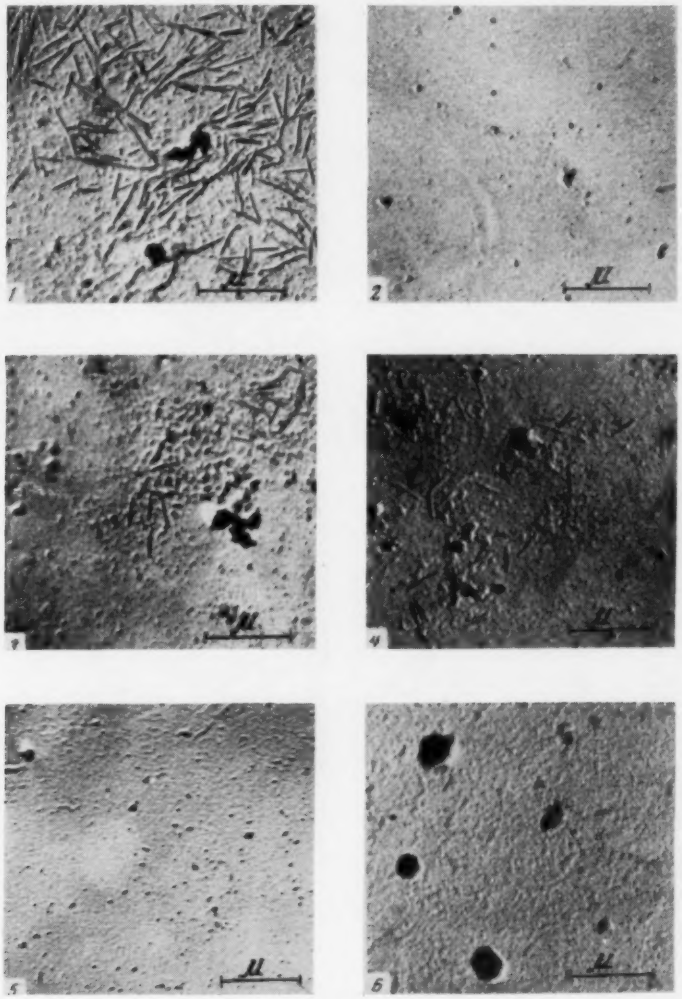

FIG. 2. Preparations of extracts of various parts of the tomato plant photographed through the electron microscope. *1*—fully developed leaves of tomato plants affected by TMV; *2*—growing points from same plants; *3*—secondary leaves from same plants; *4*—upper part of secondary leaves of affected tomato plants; *5*—growing point from healthy tomato plants; *6*—secondary leaves from healthy tomato plants. Shadowed with chromium. Dilution 1: 200.

and affected tissues were round or oval, completely black bodies which were lying free in the vacuoles. These bodies varied greatly in size; they are, apparently, droplets of tannin, which are known to be present in tomato growing points. They occurred in much greater numbers in affected specimens than in healthy plants, Bacillus-like virus particles were not seen in any of the cells from the growing point. although a large number of sections was examined.

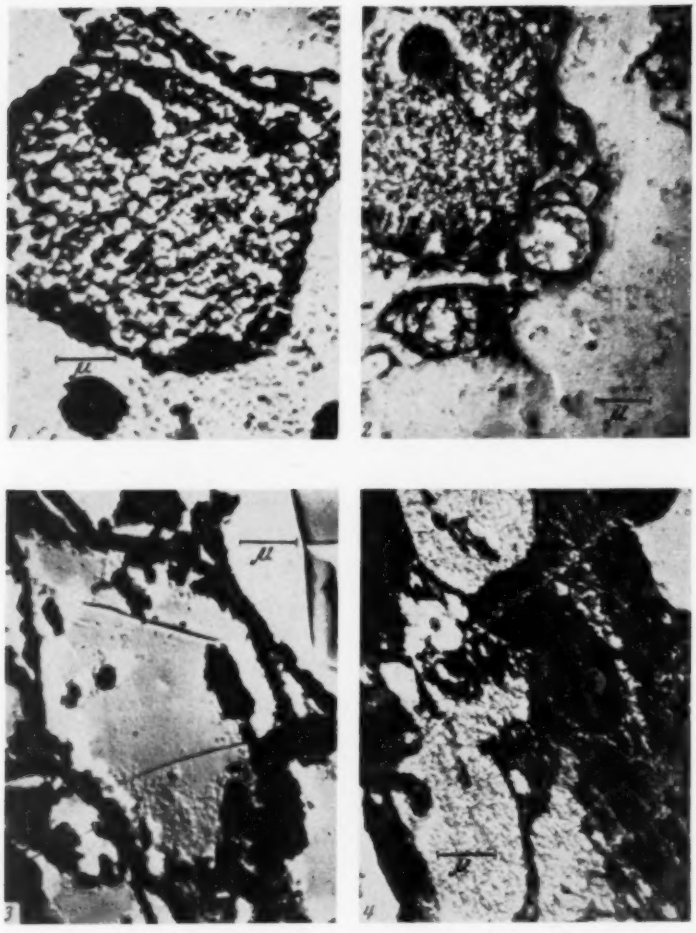

FIG. 3. Ultra-thin sections of cells from: *1*—the meristematic tissue of an affected tomato plant; *2*—the same of a healthy tomato plant cell; *3*—granularity in a cell from the basal part of the secondary leaf of a tomato plant; *4*—bacillus-like particles in cells from the apical part of the secondary leaf of an affected tomato plant. Shadowed with chromium.

It was possible to see granularity near the walls or nuclei of more differentiated cells in which the vacuole was already quite well developed. Fig. 3(1) shows part of a cell from the meristematic tissue of an affected tomato; granularity consisting of fairly large spherical bodies is visible near the nucleus. This granularity did not occur in cells from healthy specimens; Fig. 3(2) shows a similar part of the cell of a healthy tomato plant.

We were now convinced by examination of cell sections as well as by the results previously obtained that the virus does not occur in a bacillus-like form in the cells of the growing point; we therefore decided to extend our investigation to other sections, passing from immature to more developed tissues.

The most convenient subject for such examination was the growing point of the secondary leaf, which was 1·5–2 mm long. The basal part of this leaf consists of meristematic cells; the nearer the cells are to the apical part of the leaf the more differentiated they become, and the cells in the apical zone contain chlorophyll-bearing plastids.

This leaf was cut from the remaining tissues of a part which had already been soaked in methacrylate and placed in a separate receptacle, so that it would be damaged as little as possible. No bacillus-like particles occur in the secondary leaf meristematic cells, i.e. the situation was the same as in the growing point cells, so far as the virus was concerned. The cells in sections from this zone have a characteristic feature, i.e. large granular masses are present, usually along the cell walls. Isolated groups of rather larger granules occur in sap from these secondary leaf zones (Fig. 2(3)). There is a possibility that fixation and soaking in methacrylate may contribute to this, since it causes the granules to swell.

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Characteristically shaped virus particles were found only in the upper (green) part of the secondary leaves (Fig. 3(4)); this zone is shown as a dark area in Fig. 1.

A large quantity of virus is present in the upper region of the secondary leaf, and the particles are similar in appearance to the forms previously described [1, 2] in fully-developed tobacco leaves after the same fixation process. Large groups of particles are visible in which all the particles point in the same direction, in addition to the particles which lie in the cells in no particular order.

As the number of formed bacillus-like particles increases in the cells, so granularity in these cells decreases. Granularity is almost absent from the cells which are filled with clumps of these particles.

The study of ultra-thin sections of tissues from the affected tomato's vegetative gemma makes it clear that there are no bacillus-like particles of the tobacco mosaic virus in the apical meristem of an affected plant. However, they occur in the more differentiated cells.

It was also established that the formation of a large number of granular masses in the affected plant cell precedes the appearance of bacillus-like particles.

A cell zone was found which gave indications which could be interpreted as stages in the formation of tobacco mosaic virus particles [3]. In Fig. 1 this zone is shaded with vertical lines.

Translated by R. E. HAMMOND

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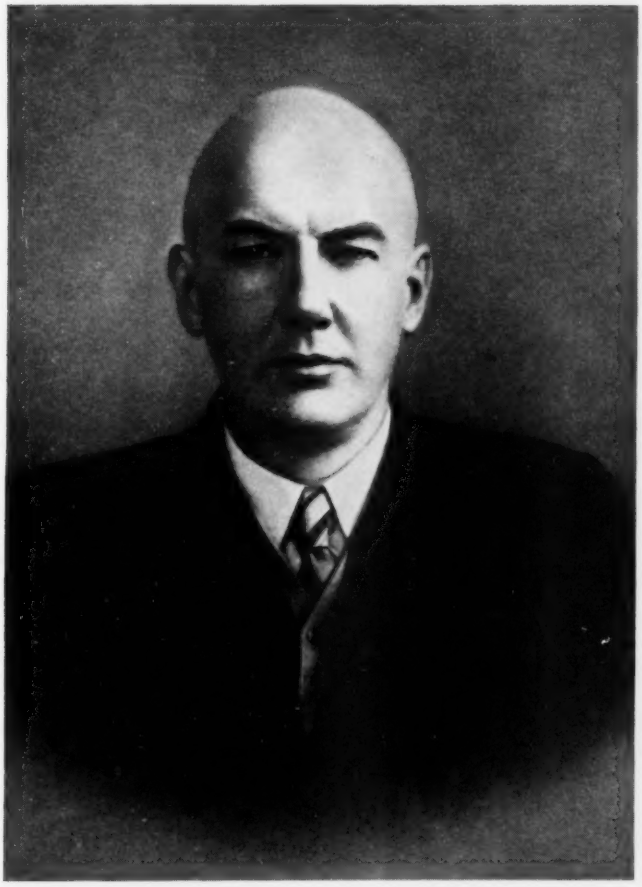

DMITRII NIKOLAEVICH NASONOV

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DMITRII NIKOLAEVICH NASONOV*
1895-1957

On 21 December 1957 the great Soviet cytologist Professor Dmitrii Nikolaevich Nasonov suddenly died at the height of his creative powers. He was a corresponding Member of the Academy of Sciences of the U.S.S.R. and an active member of the Academy of Medical Science of the U.S.S.R.

Soviet and world biological science have suffered a great loss by his death. Dmitrii Nikolaevich was the head of the largest original school of cytologists in our land, he was a gifted teacher, a transparently honest man, and supremely loyal to the Fatherland and to Soviet science.

Having started his research work studying the formation of cells in connection with the function of the organoids in them, Dmitrii Nikolaevich Nasonov and his colleagues published a large number of completed works which led to a renewed examination of the basic conditions of cell physiology.

When investigating the physico-chemical changes in cells when acted upon by various irritants, Dmitrii Nikolaevich Nasonov furthered knowledge of paranecrosis as a non-specific reaction of protoplasm to external influences and put forward the albumin theory of damage and irritation. According to this theory the basis of paranecrotic reactions is reversible denaturative changes of the cell albumins. The research work on this was referred to in the monograph by D. N. Nasonov and V. Ya Aleksandrov, "Reaction of Living Matter to External Influences" (1940), for which the Stalin prize was conferred in 1943.

Study of the effects of irritation brought Nasonov to examine a number of old theories and to form new ones to explain the rules of penetration and spread of substances in the cell (cellular penetrability), the reasons for the formation of bioelectric potentials and the origin and conduction of stimuli in the nervous and muscle tissues.

The greatest of Nasonov's achievements was his theory of gradual stimuli which proved the unity of localized and dispersed stimuli and the existence of a mechanism of self regulation of the size of dispersed stimuli in the nerve conductors and showed that the "all or none" law is only a particular case of a more general reaction. Nasonov has written about this in the monograph "Local Reaction and the Dispersed Stimulus" which is being printed by the Academy of Sciences of the U.S.S.R. Publishing House.

The experimental and theoretical works of Nasonov and his colleagues are of great value for the development of theoretical biology and medicine, and are known far beyond the frontiers of our country.

A short time ago Nasonov was entrusted with the organization of the Institute of Cytology in Leningrad by the Academy of Sciences of the U.S.S.R., and planned its further development. According to his plan, the main work of the Institute of

* Biofizika **3**: No. 3, 258–259, 1958 [Reprint Order No. B10 139].

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Cytology is the synthetic development of cytology, combining many investigations into the morphology, biochemistry, physico-chemistry ,and biophysics of the cell on a wide variety of materials for comparison from the simplest to the highest multicellular organisms.

Nasonov was not only a great scientist and teacher. He was also a courageous citizen of the Soviet Fatherland. During the Great Patriotic War he voluntarily joined the Red Army and was at the Leningrad front where he was wounded.

A shining memory of Dmitrii Nikolaevich Nasonov, the remarkable man and passionate fighter for advancement of materialist science will live among Soviet biologists for a long time.

Academicans, L. A. ORBELI E. N. PAVLOVSKI V. A. ENGEL'GARDT. Corresponding members of Academy of Sciences of the U.S.S.R. P. A. BARANOV, V. N. CHERNIGOVSKII. Corresponding members of the Academy of Medical Science of the U.S.S.R., G. M. FRANK, A. G. GENITSINSKII.

Translated by E. L. URCH

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A MODEL OF THE CHAIN CONFIGURATION IN POLY(GLYCYL-L-PROLINE)*

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(*Received* 3 *December* 1957)

STRUCTURAL studies on polyglycine and polyproline, or polypeptides deriving from glycine and L-proline, are of particular interest in relation to the chain configuration in collagen. The results are of great current interest, because it has been shown [1] that the imino acid and glycine residues concentrate in certain areas of the collagen chain. The special chain configuration in such areas can be shown to result from a specific alternation of these two types of residues. It is now supposed that a glycine–proline–hydroxyproline sequence is typically present in collagen [2–4], and a model has been built up on this basis which is in agrement with many of the experimental data [5, 6]. Some faults remain, however, the main one being that half the NH groups have no hydrogen bonds; the model cannot therefore be taken as final and further research is required.

Much information on the possible interatomic spacings and valence angles in peptide chains has been derived from work on crystalline amino acids and peptides. Stereochemical data on peptides containing proline residues have latterly been obtained [7, 8], from which the theoretically possible configurations of polypeptide chain segments containing proline rings and glycine residues can be derived. The

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results are of value in collagen studies. We here give one such possible configuration in which proline rings and glycine residues alternate.

The basic assumptions made in constructing the model were (a) the interatomic spacings and valence angles are basically the same as in simple peptides and (b) the peptide link is planar and has the *trans* configuration.

The basic structural unit assumed was glycyl-L-proline, the group containing the glycine residue and the pyrrolidine ring being assumed planar, the proline bond being taken as lying at 53° to the plane (Figs. 1, 2). The plane passing through the

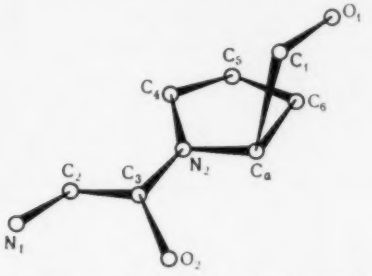


FIG. 1. Structural scheme for glycyl-L-proline.

Cα–C_1 and C_1–O_1 bonds in the proline can rotate about the C–C_1 bond in the proline residue. The angle at which the plane is set determines the mutual positioning of two successive planar groups, i.e. the configuration type. The poly(glycyl-L-proline) chain thus only bends at the Cα points, so the configuration is ultimately determined by the stereochemistry of the proline residue.

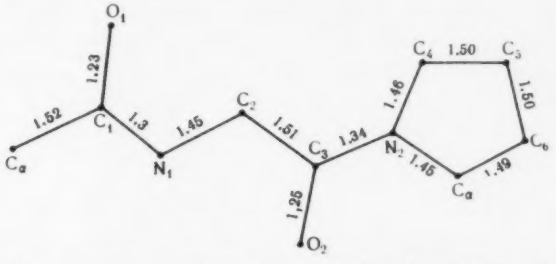


FIG. 2. The planar group which forms the structural unit of the model. The atomic separations are given in the figure. The valence angles are:

$<C\alpha C_1N_1$	= 115·3°	$<O_2C_3N_2$	= 125°
$<C\alpha C_1O_1$	= 121·4°	$<C_4N_2C\alpha$	= 113°
$<O_1C_1N_1$	= 123·3°	$<N_2C_4C_5$	= 104°
$<C_1N_1C_2$	= 122·2°	$<C_4C_5C_6$	= 105·6°
$<N_1C_2C_3$	= 114·5°	$<C_5C_6C\alpha$	= 107°
$<C_2C_3O_2$	= 116·8°	$<C_6C\alpha N_2$	= 103·7°
$<C_2C_3N_2$	= 118·2°		

The possible configurations which can be constructed on this basis include an interesting spiral one. The spiral is left-handed, with 3·3 residues per turn, or 10 per 3 turns, the pitch being 9·6 Å. The projection of a group on the spiral axis is 2·9 Å, the planar groups lying at ~60° to the axis (Fig. 3). In each group one CO and one NH bond is directed within the spiral, and one CO bond is directed outwards;

the CO bonds lie at 20° to the horizontal plane. The spiral radius (including the pyrrolidine ring atoms) is 5·6 Å, and the configuration is such that there must be a

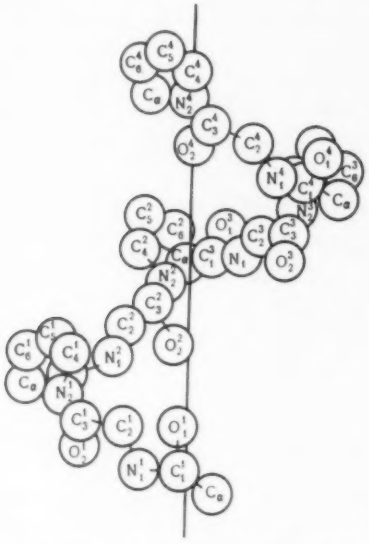


FIG. 3. The poly(glycyl-L-proline) chain.

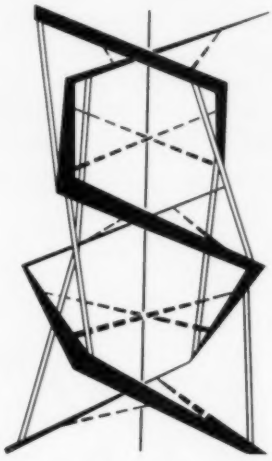


FIG. 4. Combination of two polypeptides chains, the intramolecular hydrogen bonds being shown dashed. The double lines represent water bridges.

tendency for a stable complex between two such spirals to form. The complexes can be formed by hydrogen bonding between $N^1{}_1$ and $O^2{}_2$, and $N^2{}_1$ and $O^1{}_2$, which hydrogen bonds are almost normal to the spiral axis (Figs. 4, 5). The two spirals are so placed that a two-fold axis directed along the fibre axis results. As before, the radius of the resulting "molecule" will be 5·6 Å, the calculated density being 1·4 g/cm^3.

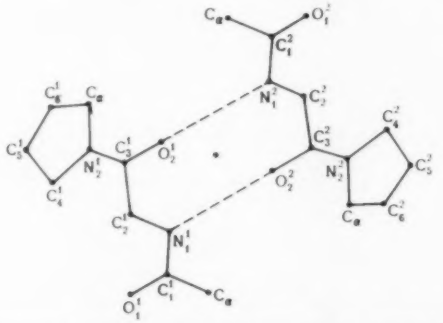


FIG. 5. Method of linking the residues in two chains. Projection on a plane normal to the spiral axis, the hydrogen bonds being shown dashed.

The approximate atomic co-ordinates have also been calculated (Table 1); as the model is a spiral with 10 residues per 3 turns the diffraction pattern will show a general resemblance to that of collagen [9], i.e. will show strong maxima at about the tenth

layer line ($\sim I_0$), corresponding to the meridional $d = 2{\cdot}9$ Å repeat, and at the twentieth layer line ($\sim I_0$) (second order for $d = 2{\cdot}9$ Å); near-medional maxima on the seventh layer line ($\sim I_1$) corresponding to $d = 4{\cdot}1$ Å, and finally maxima on the third layer line ($\sim I_1$) corresponding to the fibre axis repeat period of 9·6 Å. The structure factor has also been calculated for the zero layer line (Fig. 6); the minima on this curve correspond to intensity minima on the equatorial layer line of collagen. This resemblance between the two patterns enables us to discuss our model as the basis of the areas in collagen chains which have the specific collagen configuration.

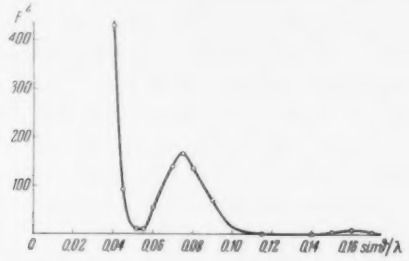


FIG. 6. Calculated structure factor for the zero layer line.

TABLE 1. CALCULATED ATOMIC CO-ORDINATES

Atom	r(Å)	$\theta(\psi)$	z(Å)
Cα	4·1	0	0
C_1	3·8	19	0·7
O_1	4·8	28	1·1
N_1	2·6	29	0·9
C_2	3·0	53	1·6
C_3	2·7	82	1·9
O_2	1·7	104	1·5
N_2	3·8	88	2·6
C_4	4·9	77	3·0
C_5	5·6	89	3·6
C_6	5·0	103	3·7
Cα	4·1	108	2·9

Grassman [1] has recently given some interesting data on the chemical structure of collagen, namely that glycine and proline predominate in certain areas of the chain; peptides containing 30–40 residues of these acids were separated, with a 1 : 1 ratio between the acids; in some cases the glycine was slightly in excess. Grassman's *sf* peptide, showing the sequence H_2N-gly-(ser, thr, leu, ala, gly)-[10 pro, 5 hypro, 12 gly, 2 ala, 1–2 ser, 2 tyr, 1 glu]-phe-glu-leu-asp-COOH, is of particular interest. The number of imino acid residues in the central area (square brackets) to the number of glycine + alanine residues was 1 : 1*.

These results fit in perfectly with our views on the collagen structure [10] derived from our X-ray studies on collagen group proteins; we observed intensities

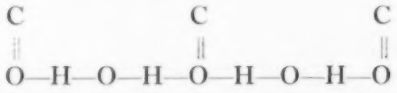
* Glycine and alanine residues are assumed to be stereochemically equivalent.

corresponding to $d = 2{\cdot}9$ Å and 11 Å which were dependent on the glycine and imino acid contents. The relationships for both acid species were straight lines, both lines being almost the same. We therefore concluded that these spots were due to areas of the collagen molecules containing imino acid and glycine in a 1 : 1 ratio, and from this ratio we deduced that there was a reasonable probability of a gly-pro-gly-pro sequence in such areas (proline and hydroxyproline being interchangeable). A gly-pro-hypro sequence was also considered probable in some areas, but this would imply an imino-acid : glycine ratio of 2 : 1, which is not found. Some structures proposed by Huggins [5, 6], of gly-pro-(any amino acid) type, are not considered likely, as these structures were deduced from the overall composition. Since the specific configuration only occurs in isolated areas the most probable sequence should be chosen from the composition of those areas alone, and we consider the specific sequence of residues in these areas to be responsible for their stereochemical peculiarities; the areas do not correspond in configuration to the equilibrium forms of most proteins. The collagen group proteins form a distinct structural group, and our model is one of the possible ones for isolated areas of the collagen molecule.

Our model agrees with all the experimental data on which the earlier models have been tested, namely, it gives the observed CO and NH dichroism in the infra-red and it agrees with the X-ray data. It indicates that the collagen molecule consists of denser (ordered) and less dense areas; the density alternation may explain why density variations along collagen fibrils are seen in the electron microscope. It also explains the observation of Orekhovich and Shpikiter [11] that the collagen molecule consists of two components bound together by hydrogen bonds. We have already pointed out that our model would predict stable combination of two chains.

Our model also explains why the collagen chain is only slightly extensible, and why collagen gives no β-configuration. Since our chain can only bend at the proline Cα it can only straighten out by rotation in the Cα–C_1 bonds; these bonds can only take up two orientations relative to the pyrrolidine rings, since any other configuration distorts the valence angles. The Cα–C_1 bonds therefore cannot rotate, so the spiral cannot be extended. The range within which the valence angle at the α-carbon in proline can vary is unknown, but it is clearly insufficient to allow the chain to stretch and take up another configuration. The chain can, however, coil and give statistical coiling by free rotation about the proline α-carbon.

It is also possible to fit water molecules into the model in the way required by our data on collagen hydrates [12]. Some of the CO-groups are free, because the pyrrolidine ring N atoms do not participate in hydrogen bonds; they can then give rise to water bridges, as in the diagram:



continuous spirals of water molecules being formed (Fig. 4). The O atoms in the H_2O molecules lie about 3·5 Å away from the chain axis, the distance between successive H_2O oxygen atoms, projected on the spiral axis, being about 2·9 Å. The resulting structure agrees with the intensity changes found in the X-ray patterns on altering the water contents of collagen preparations [12].

The collagen structure becomes imperfect as water is removed [12]; our model also explains this, because when some water is removed some of the CO groups remain bound to one water molecule only, and this produces strains and distortions in the chain. Our model is a possible one, and would appear to be one of the most probable, but further experimental studies on such polypeptides are required to determine whether the structure is identical with that of the special areas in collagen.

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LIGHT- AND DARK-FIELD ELECTRON-MICROSCOPE STUDIES ON THE CHLOROPLASTS OF *BELLIS PERENNIS**

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BY fixing and microsectioning chloroplasts for the electron microscope it has been possible to derive detailed structural data, and it has been shown that in higher plants the chloroplasts consist of stroma plus included grains, and of osmiophilic granules. The stroma contains thin lamellae, which extend from one grain to another; the grains consists of discs. The chloroplasts have outer membranes [1]; Strugger [2] demonstrated that the chloroplasts in *Chlorophytum comosum* leaves had double boundary layers, and also states that, within the chloroplasts, the periphery differs from the rest by having no grains or lamellae, but having obscure globular structures, apparently of liquid nature. He considers that this part corresponds to Senn's "peristromium" (observed in the light microscope) [3, 4], but unfortunately gives

* Biofizika **3:** No. 3, 1958, 265–268 (Reprint Order No. B10 141).

no photomicrographs to supplement the descriptions. Muhlethaler [5] has raised no objection to these observations, though the chloroplast membrane has not always been found [6, 7].

The structures of intact chloroplasts are somewhat difficult to study, because the chloroplasts are rather thick* and consist of high-density elements, though some regions are so thin that their contrast against the film backing is too slight to reveal them in the light-field picture. Regions differing little in thickness are detectable in dark-field electron-microscope methods,† but the intensity distribution in a dark-field image cannot be unambiguously referred back to thickness differences [9, 10], since two regions of different thicknesses can give the same plate blackening. This effect becomes more marked at higher voltages, so the least possible accelerating voltage should be used in dark-field studies and, to avoid ambiguities in interpretation, one should compare the dark-field images for different voltages with the light-field ones. Studies on intact chloroplasts are of considerable value, so dark-field methods should be used; in our work we used *Bellis perennis* and light and dark fields.

METHODS

The specimens were prepared as previously [11], *Bellis perennis* leaf samples 2·5 mm in diameter being punched out. Ten such samples were placed in a porcelain mortar and 1 ml of 1% OsO_4 in sucrose buffer (acetate/veronal pH 7·4 buffer, concentration 0·28 M; sucrose 0·4 M) added as fixative, and the material worked with the pestle until a homogeneous suspension was obtained. The mixture was left for 2 hr in darkness at 4–6° C to fix, and the chloroplasts washed by drop dialysis [12]. A UEM-100 electron microscope was used, the dark field being produced by displacing the aperture stop normal to the optical axis, so all unscattered electrons were cut out. Light- and dark-field photographs were taken at 40, 60, 80 and 100 kV, a 30 μ diameter stop being used.

RESULTS

The *Bellis perennis* chloroplasts were found to be rounded bodies 3–6 μ in diameter and, being very dense, scattered electrons strongly. The chloroplasts differed in electron transparency. The arrangement of the osmiophilic granules at the chloroplast edges is notable, as some are in contact with grains, while others give the impression of being particles not bound to the chloroplasts (Figs. 1c, 2a). Areas of lower thickness can sometimes be seen at the edges of chloroplasts (Fig. 1a and c). At 40 kV the internal structures could not be distinguished (Fig. 1a), the electron transmission increasing with voltage, though the contrast fell. At 80 and 100 kV the peripheral structures are well seen, and sometimes the central ones, but some chloroplasts were so thick that their internal structures could not be distinguished even at the higher voltages (Fig. 1c). In the dark-field studies the central structures were seen in the thick chloroplasts (Fig. 1b and d,‡ and the grains and osmiophilic

* By "thickness" we hereafter mean effects due to thickness proper and/or density.

† The minimum thickness detectable by dark-field methods is an order of magnitude less than for light fields, even for objects of low Z [9].

‡ The dark-field resolution is below that of the light-field, being 100–200 Å for amorphous materials.

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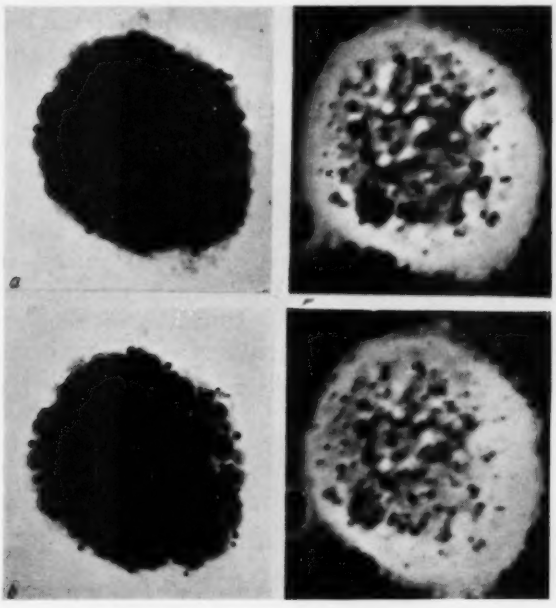

FIG. 1. Microphotographs of the chloroplast of *Bellis perennis*. (a) Light-field, V = 40 kV; (b) Dark-field, V = 40 kV; (c) Light-field, V = 80 kV; (d) Dark-field, V = 80 kV.

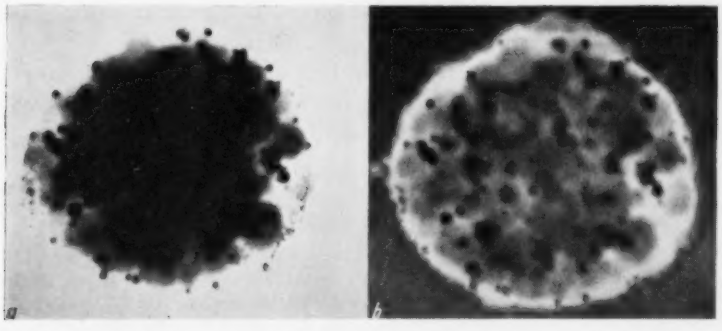

FIG. 2. Microphotographs of the chloroplast of *Bellis perennis*. (a) Light-field, V = 60 kV; (b) Dark field, V = 60 kV.

granules are clearly visible. The osmiophilic granules between grains are visible in the light-field images, but the dark-field pictures show that they are distributed over the whole chloroplast, since the grain and granule images may overlap (Fig. 1c and d). The grains differ in density. The dark-field images of chloroplasts of various thicknesses show edge zones which cannot be distinguished in the light-field image; these zones are grain-free and contain osmiophilic granules (Figs. 1b and d; 2b), and 0·15–0·2 μ wide; the granules lying at the edge evidently fall entirely in this zone. The dark-field intensity distribution shows that this peripheral zone is thin;

some chloroplasts show another layer outside this zone, of width 0·25 μ. Parts of this layer can be seen in Fig. 1b and d, but are scarcely distinguishable in the light-field (Fig. 1a and c); the layer is clearly extremely thin. The dark-field image is unchanged in character between 40 and 80 kV and corresponds to the light-field image (Fig. 1); this indicates that the physical characteristics of the chloroplast are uniquely related to the intensity distribution in the image plane, i.e. the structural elements seen in dark-field images are real.

The unscattered electron flux gives a background which diminishes the contrast of the light-field image, and this restricts the detail which can be seen if the thickness differences are small; whereas in the dark-field image the unscattered electrons are blocked out by the aperture stop and the contrast is much higher. The dark-field studies on *Bellis perennis* show why the granules, which appear in the light-field image to be separate from the chloroplast, are not scattered about; they are bound to very thin structural elements which cannot be detected in the light-field image. The exterior layer, partially visible on the photomicrographs (Fig. 1), may possibly be part of the membrane.

Dark-field methods also give us certain additional data on the very thick structural elements, since the structure of the thick central area of the chloroplast, the grain arrangement and the osmiophilic granules are all seen at all voltages. The dark-field image also shows more clearly structural elements which can be seen in the less thick chloroplasts in the light-field image. Certain additional information can thus be obtained by using both methods simultaneously.

SUMMARY

(1) Dark-field studies reveal a thin edge zone which contains osmiophilic granules in all *Bellis perennis* chloroplasts, of whatever thickness.

(2) The structure of the central area in thick *Bellis perennis* chloroplasts, which is not distinguishable in the light-field image, can be seen in the dark-field one.

We are indebted to V. A. Smirnova and I. G. Stoyanova for valuable discussions and advice.

Translated by J. E. S. Bradley

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QUANTITATIVE CYTOCHEMICAL RESEARCH ON RIBONUCLEIC ACID IN THE GANGLIAL CELLS OF NORMAL EXHAUSTED RETINAS*

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When working on ribonucleic acid (RNA) in nerve tissue the research in various functional conditions of neurons is particularly important. However, the experiments were so planned, using hyperfunction, that the state of nerve cells [1–4] under conditions of exhaustion were the most frequently studied. One set of tests were made on the changes in RNA during normal neuron function with adequate irritants. Brattgard [5–6] noted that RNA disappeared totally from ganglial cells of the retina of rabbits 3 hr after the animals had been kept in the dark. Other authors [7] found no changes in the quantity of RNA in the adaptation to light and darkness in the cells of the retina of rabbits.

The retina is very convenient in the study of chemical changes in various functional conditions of the nerve cells by cytochemical methods. Retinal cells react to light which is their natural irritant and do so in such a way and by such an extent that can easily be measured. The retina is studied in detail in many physiology tests in connection with research into the process of sight. The various physiological indicators of the condition of retinal cells are of great interest to the cytochemist. Finally, the short time required to fix or treat an eye histologically is important for cytochemical research, and lessens the possible errors in the conclusions. We therefore chose the retina for study of RNA changes in the functional condition of the nerve cells. We started by studying the changes in RNA when the retina adapts to light or darkness. We used the retina of the frog *Rana ridibunda*. The fixation was done in summer. By ultra-violet cytophotometry we found that the quantity of RNA in the cytoplasm of ganglial cells exposed to 6 hr uninterrupted light was about twice as much as in the same cells left for the same length of time uninterruptedly in the dark. At the same time we found that the quantity of RNA in cytoplasm does not depend on the time of the day but only on the conditions of illumination. The average optical density of RNA to light is $0{\cdot}35 \pm 0{\cdot}02$ by day and $0{\cdot}35 \pm 0{\cdot}05$ by night (i.e. the same by night as by day). In darkness, by night it is $0{\cdot}18 \pm 0{\cdot}009$ and by day $0{\cdot}16 \pm 0{\cdot}009$. A lowering of the quantity of RNA can show a lessening of the rate of synthesis of the albumin in the cells adapted to darkness. Actually in animals remaining in darkness over a long period of time the weight of ganglial cells lessens [5, 6] and the rate of reactions to the SH-groups of albumins lessens [8].

* Biofizika **3**: No. 3, 269–273, 1958 [Reprint Order No. B10 142].

In the papers referred to, it was not made clear how soon the content of RNA changes when the functional conditional of ganglial cells changes, nor whether the process coincides in time with the adaptation of the eye to changes in the conditions of illumination. Research into RNA with various functional loadings of the neurons and at the time of their exhaustion would be no less interesting. The aim of our present work was to clarify these questions.

We studied the RNA content with the aid of an ultra-violet microscope. The ganglial cells of the retina of the frogs *Rana temporaria* which had been in the dark were examined after 24 hr. They were given a burst of light for 3 sec and examined after 0·5, 2, 10 and 30 min and after 2, 6 and 24 hr. Interrupted light was found to be a more powerful irritant than a constant light. Some frogs were put in the dark for 6 hr after 24 hr in the light. The eyes were fixed under the same conditions as the test—in the dark or under interrupted light. We used formalin–spirit–acetic acid in quantities of 3 : 1 : 0·3 as a fixing agent [9]. The prepared retina was put

Fig. 1. Microphotograph in ultra-violet rays (265 mμ). Objective 58 × nA = 0·8. Eyepiece × 8. Magnification × 1300. The nucleic acids in ganglial cells of the frog retina in darkness and light; (a) darkness, 24 hr; (b, c and d) intermittent light; (b) 2 hr, (c) 6 hr, (d) 24 hr.

in paraffin wax. All measurements were on sagittal sections, 6 μ thick, across the optic nerve as it leaves the eye. The fixation was done in winter.

When observed in the ultra-violet microscope the cytoplasm of ganglial cells of the retina was seen to be a narrow edging around a large nucleus. In animals which had been in darkness for 24 hr the cytoplasm of the nerve cells of the retina absorb ultra-violet light very feebly. In most cells the structure of the nucleus (see Fig. 1a) is clearly visible. As a result of light irritation the number of substances which absorb ultra-violet light increases considerably (see Fig. 1b, c). In cells which had been adapted to darkness these showed up pale grey on the ultra-violet microscope, but after 2–6 hr of illimination we found intensely black cells whose structure was indistinguishable because of the great absorption. After 24 hr illumination the absorption of ultra-violet light gets less and the structure of the cytoplasm and the nucleus are noticeable.

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Ultra-violet light of 265 mμ is absorbed by nucleic acids and aromatic amino acids. In order to limit these substances and to determine the RNA quantitatively the method of ultra-violet photographic cytophotometry was used [10, 11]. This method allows determination of the concentration of a substance with a 10 per cent accuracy. The measurements could be taken both in separate structures and on sections of the cytoplasm or the nucleus. The ganglial cells were easily located, and their average measurements in all variations of our test were practically the same. Bearing this in mind we limited our measurements by determining the concentration of RNA which in this case was equivalent to the whole quantity of the tissue.

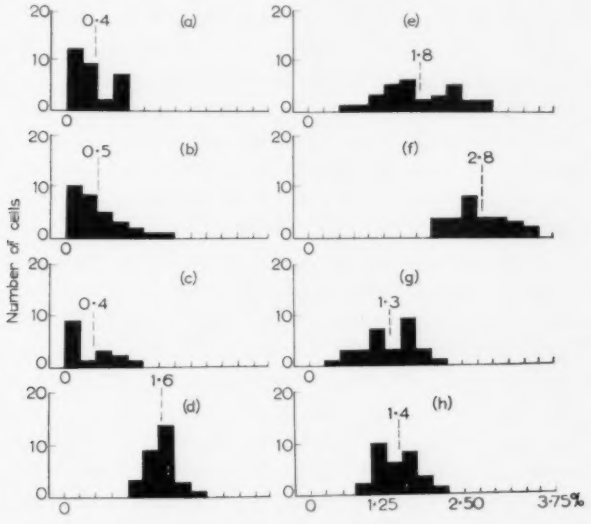

FIG. 2. Graph of concentration of RNA in the cytoplasm of individual ganglial cells of the retina. Ordinate: the number of cells. Abscissa: concentration of RNA (per cent). Dotted line with a number over it shows average concentration of RNA.
(a): darkness for 24 hr. (b): interrupted light 2 min (c): interrupted light 10 min (d): interrupted light 30 min (e): interrupted light 2 hr (f): interrupted light 6 hr (g): interrupted light 24 hr (h): 6 hr darkness after 24 hr light.

The results of our measurements are presented in Fig. 2. The concentration of RNA in the cytoplasm of ganglial cells adapted to dark is considerably lower than in cells adapted to light. However, even after 24 hr in darkness, the RNA in the nerve cells does not disappear altogether. Thus when there is a considerable or almost total drop in activity of the retina, measurable quantities of RNA (with an average concentration of 0·5 per cent) persist in the ganglial cells. After 2 min and 10 min the concentrations of RNA and the appearance of the histograms (see Fig. 2) have not changed, although the adaptation of the eyes to light, as we know, has occurred by this time. But the shift of pigments in the offshoot of epithelium which is an indication of adaptation occurs 2 min after the start of illumination. An increase in the concentration of RNA occurs between 10 and 30 min, i.e much later than the ganglial cells start their intense activity. From 10 min to 2 hr after the beginning of light acting as an irritant, the concentration of RNA was 4–5 times what it was when the cells were adapted to darkness. After 6 hr the concentration of RNA is still higher, having an average concentration of 2·8 per cent. Thus over a time of illumination comparable to the normal daily functioning of eyes the concentration of RNA grows uninterruptedly. With considerable tiring of the eyes (using intermittent light for 24 hr) the quantity of RNA in the cytoplasm suddenly drops to about half what it was after 6 hr illumination. Swedish authors [1–3] refer to a drop in concentration of RNA where the nerve cells have been overworked. As is shown in Fig. 2, a return to the initial concentration of RNA does not occur when the animals are in darkness for 6 hr following 24 hr of interrupted illumination.

Our findings allow us to conclude that the quantity of RNA in the cytoplasm of the retina depends on the intensity of the function of the ganglial cells. Under normal physiological loads the average quantity of RNA rises in comparison with the condition of relative quiescence, and up to a determined limit (see Fig. 2) the quantity of RNA grows with the time of light stimulation. This observation can be used when analysing variations of quantities of RNA within the same kind of group of nerve cells. As seen in Fig. 2, in variations of the test certain cells responded differently to the stimulus. In histograms which characterized a number of cells with one or other concentration of RNA, it was obvious that whatever happens (darkness, intermittant light) some cells are distinguishable by their quantity of RNA. In animals 30 min to 2 hr in the light, a number of the ganglial cells contain as much RNA as some more basophilic cells in animals adapted to darkness. The number of such cells is only 10 per cent. After 6 hr of illumination all ganglial cells we tested contained considerably more RNA than the cells of the retina adapted to darkness, but in the first group of cells there were variations in RNA content. Changes of RNA observed in the nerve cells of the retina are also peculiar to other neurons of the central nervous system. Hyden [1] irritated the spinal motor nerves with a mild current and observed an increase in RNA; on the other hand when the same cells had been exhausted there was a drop in the RNA content. One of us made a study of the variability of content of RNA in the motor nerve cytoplasm during various functional conditions of the spine [8, 12]. Retinal cells and spinal nerves and probably other neurons do not work simultaneously or at the same intensity. This may explain the variability of concentration of RNA in their cytoplasm. In the absence of function, with a considerable drop in intensity, or in exhaustion the quantity of

RNA drops. There is an essential difference in the condition of the RNA in these processes. In the non-tired cell the amount of RNA increases relatively quickly under normal working conditions, but in the exhausted cell the RNA changes are slowed down.

At the same time it follows from our tests that the changes in RNA, when the functional state of the retina is altered by light or darkness, are not the primary ones. RNA changes in content considerably more slowly than the ganglial cells adapt to light and initiate their highly sensitive function. Evidently RNA does not directly take part in the specification of nerve cells although judging by the regular changes in RNA it probably ensures some of the processes associated with the action of neurons.

It is important to note that, even after 24 hr in darkness, RNA is still present in the cytoplasm of ganglial cells. This can be explained by the synthesis of, or renewal of, albumin which produces RNA and occurs under various conditions of neuron activity, including relative quiet. We mentioned the direct relationship between the amount of RNA and albumin in the nerve cells in an earlier article [9]. The increase of quantity of RNA according to neuron activity can be explained by a rise in synthetic processes which compensate for the growing "wear and tear" of the albumins. If the degree of intensity of these processes can be judged according to the quantity of RNA in the cytoplasm the increase in the level of albumin synthesis occurs fairly quickly, after 30 min, or even a little sooner. Thirty minutes after the beginning of the highly sensitive action of the neuron, the quantity of RNA in its cytoplasm has increased several times. This indicates great lability of RNA in nerve cells.

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INTRACELLULAR RECORDING OF MUSCLE FIBRE POTENTIALS USING REPEATED STIMULATION*

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IN an earlier article [1] we described the results of intracellular recording of electric potentials of muscle fibres with single direct and indirect stimuli. These results agree with the findings of other research workers and allow the properties of the process of excitation in muscle tissue to be explored in greater detail. But in natural muscle activity we have to deal not with a single excitation but with a large number of successive waves. By using intracellular recordings we can also in this case analyse electrical changes in individual muscle fibres and explain the peculiarities which distinguish the rhythmic action of muscles from a single excitation.

METHODS

The experiments were made on sartorius muscles of frogs. The preparation of the microelectrodes, their introduction into the fibre, and the recording and amplifying of the potentials [1, 2] were described by us earlier.† The main difficulty when recording rhythmic potentials intracellularly is that when the muscles contract there is a rapid breakdown of the electrode which slips out of the muscle fibre, or the latter is damaged by the microelectrode. To some extent, this can be avoided by careful fixing of the muscles and by using a flexible connection between the microelectrode and the input terminal of the recorder. The intactness of the electrode tip and its position in the centre of the muscle fibre can be easily determined after inducing tetany by recording an unchanged membrane potential and resistance of the microelectrode. Damage to the muscle fibre can be recognized by the peak potentials rapidly becoming progressively weaker and eventually becoming localized.

A generator of rectangular impulses was used for the stimuli; it had a transformer or an ordinary induction apparatus at its outlet. It cuts out the polarization of the stimulating electrodes and the preparation found in prolonged tetany by impulses of direct current, and also prevented grounding of the preparation through the stimulator.

RESULTS

Double stimulation. The peak potentials (p.p.) in double stimulation depended on how far the microelectrode was from the origin of the impulse in a given muscle fibre. If the distance between the area for distributing stimulating electrodes in a

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† Reference [1], when describing the microelectrode in connexion with its penetration of the cell and the possible degree of changes in its own potential, contains an error: it should read 0·8 mV.

direct stimulus (or an end plate in an indirect one) and the point of introduction of the microelectrode was a few millimetres the second p.p. suffered the usual changes connected with the formation of absolute and relative refraction after the first impulse. The duration of the absolute refractory phase in the basis mass of muscle fibres varied from 3·7–5·0 msec, which corresponds approximately to Serkov's findings on isolated muscle fibres of the same muscle [3]; duration of the relative refractory phase was 10–12 msec. By measuring the speed of conduction of the stimuli in the tissues mentioned [4], we found it varied from 2·5–4 msec; but fibres were also met with which were characterized by a considerably lesser speed of conduction, from 0·8–1·5 msec. In such fibres the duration of the period of absolute refraction reached 10 msec and more and the duration of the relative refractory period was 30 msec and more. In Fig. 1 we give some examples of p.p. of muscle fibres with double stimulation.

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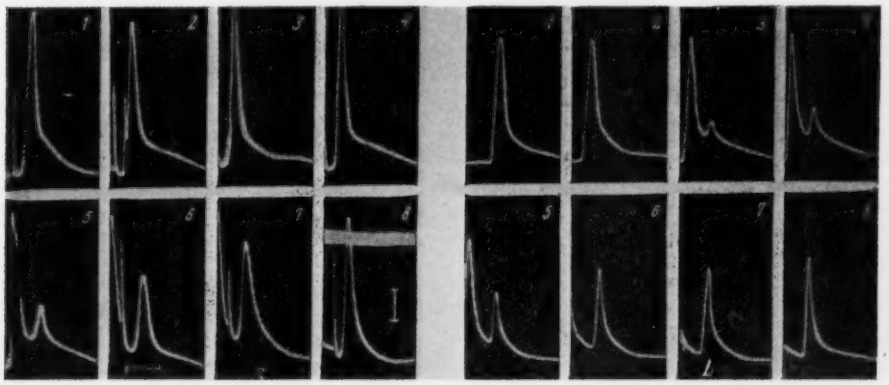

FIG. 1. Peak potentials of muscle fibres with double stimuli. (a) Curarized muscle, direct stimulus, recorded 1 cm from the origin of stimulus. (1) one stimulus. (2)–(8) two stimuli with intervals of 3, 3·7, 4·5, 5·2, 6·1, 7·5, 15 msec. (b) Normal muscle, indirect stimulus, recorded in the area of innervation. (1) one stimulus. (2)–(8) two stimuli with intervals of 4·5, 6, 7·5, 9, 10·5, 12, 13·5 msec. On this and following figures the effects of the second stimulus are in the same sub-picture. The effects of the first stimulus are shifted forward. Scale: 25 mV and 10 msec.

In recording the p.p. furthest from the origin of the impulse, we found it impossible to determine the changes associated with the relative refraction in the size of the second p.p. The second p.p. appeared initially with a larger interval between the stimuli than is described in the case above, and immediately after with almost the maximum size. Consequently the impulse arising at the beginning of the relative refractory phase subsided quickly. Only the more powerful impulses arising at the later periods of relative refraction had a sufficient depolarizing action on the nearby fibres to spread further. The second wave of stimulus, weakened by the relative refraction, spread much slower than the first [4]. As the speed decreased, the second wave lagged behind the first and escaped the zone of relative refraction and then recovered its strength till it reached its maximum size (Fig. 2).

If the weakened impulse spread and recovered its usual intensity, the size of its

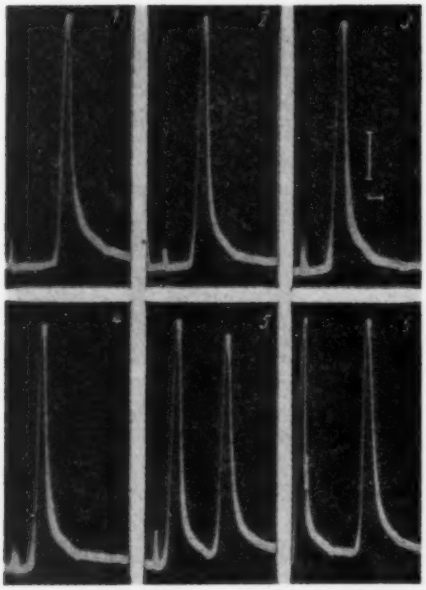

FIG. 2. Peak potentials of muscle fibres. Curarized muscle, direct stimulus. Recording at 2 cm distance from the site of stimulation. (1) one stimulus. (2)–(6) two stimuli with intervals 5·2, 6, 7·5, 9, 15 msec. Scale: 25 mV and 5 msec.

p.p. was nevertheless somewhat less than its maximum (about 5–6 per cent). This change, in distinction from the reduction caused by relative refraction, only very slowly weakened as the interval between the stimuli lengthened and was sometimes noticeable when the intervals were up tc 100 msec.

Local potentials. The basic difference of local potentials (l.p.) with double stimuli was the absence of refraction and their ability to summate together even with the shortest intervals between two nerve impulses. Our findings fully conicide with the results of other research workers obtained both by extra- and intra-cellular recordings [5–11].

The second l.p. of an end plate could be obtained with an interval between nerve stimuli of about 2·5 msec. Since the duration of absolute refraction of motor nerve fibres in the frog's nerve is 1–5–3 msec, it is clear that the nerve ending can produce a l.p. even when a nerve impulse of minimum intensity originates in the nerve fibre. It is possible both in the nerve and in the muscle fibre that the impulse, weakened by refraction, becomes stronger in delayed conduction and recovers its normal intensity [12]. However, no significant increase of the latent period of the second l.p. can be seen, as should be the case if this mechanism were the only significant one involved. Evidently the determining factor of synapse transmission in neuro-muscular combination is sufficient to make an l.p. possible even by a weakened impulse.

When a second l.p. was superimposed upon the l.p. from the first impulse, the summary depolarization of the end plate increased, and was often sufficient to

overcome the block to neuro-muscular transmission and to spread the impulse throughout the fibres. The second l.p. in a short period of time also became spontaneously more powerful than it was initially (Fig. 3b). It is true that the degree of increase of the second l.p. was not uniform; it was most marked with mild curarization and was almost totally absent when the curarization was deep (Fig. 3a). The increase of the second l.p. was maintained when this l.p. overrode the previous l.p. at its descending curve over a period of 15–20 msec. The maximum degree of increase was 25–30 per cent of the initial size of the l.p.

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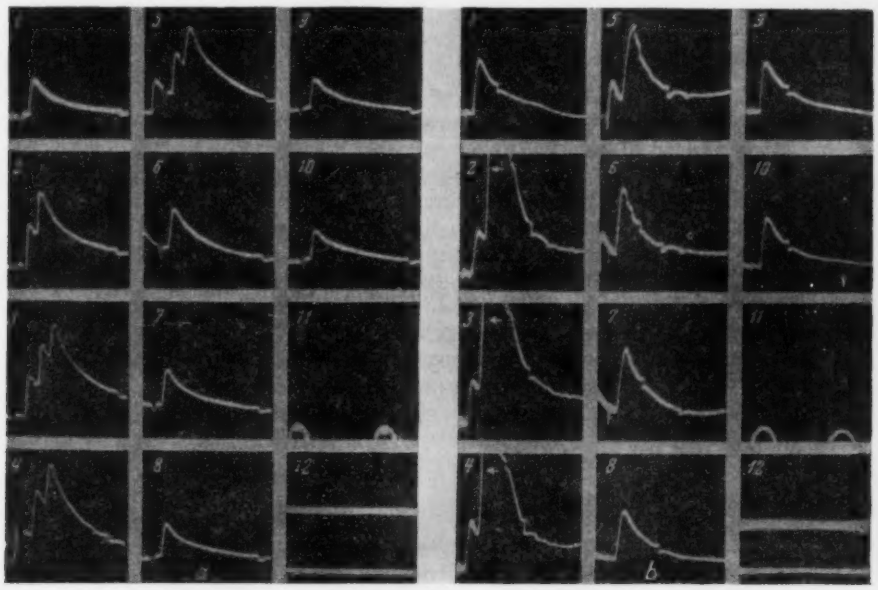

FIG. 3. Local potentials of the end plate with double indirect stimuli. (a) Deep curarization. (1) single stimulus. (2)–(10) two stimuli with intervals of 2·5, 3, 4·5, 6, 15, 30, 45, 75 msec. (11) 50 c/s; (12) 30 mV. (b) Same, with curare washed off.

If after mild curarization the l.p. originated a peak process as a result of summation, this would start from the highest point of the second l.p. In such cases its going over to the peak could only be recognized with difficulty by a small bend in the ascending curve (Fig. 3b). The apex of the p.p. on this occasion went far beyond the limit of observation, and the probable place where the l.p. went over to the p.p. is marked with an arrow.

If the degree of curarization was deeper, the time of appearance of the p.p. was delayed. The p.p. was not at the highest point of the second l.p. but on its descending curve. Kuffler has referred to this earlier [13]. Often the p.p. lost its height and changed to a small potential wave similar in character to the l.p. (Fig. 3a).

If the interval between two stimuli was increased so much that a second l.p. occurred after the first had been completed, a certain depression of the second l.p. could be seen. In contrast to the increase described above, the depression was also well marked with deep curarization. In the depression period the l.p. could become

20–25 per cent weaker. There was a well marked weakening even when 75–100 msec intervals occurred between the stimuli.

The formation of a local stimulus in the end plate was thus accompanied not only by corresponding electrical changes but also by a characteristic two-phase change in the effectiveness of the second nerve impulse. This is also characteristic, as we know, of other synaptic junctions. In the neuro-muscular synapse, an exact solution of the question of localizing the changes mentioned made more difficult because both impulses always pass across the same synaptic termination. An analogous depression at synaptic junctions in motor neurons is often attributed to presynaptic changes, while data on the presence of it only in homosynaptic, or its absence in heterosynaptic, sequences of impulses as shown by Eccles [14] are advanced as proof. In this case some authors observed a clear depression also when applying two weak impulses to the cell through various synaptic endings [15–17]. Of course, under such conditions localization of the impulse can only be post-synaptic. Other research workers [12] think that the period of elevated effectiveness of the synaptic action is also connected with changes which are localized presynaptically. However, if the l.p. occurred in the muscle fibre not because of synaptic action but as a result of a direct stimulus, or of upsetting the conduction of a maximum impulse which was spreading along the muscle fibre, then one could also observe a rise in the effectiveness of the second l.p. if it fell on the descending part of the first one.

One should stop and consider the singular protracted l.p. which we succeeded in recording from some muscle fibres [1]. When combining two impulses, the changes of such a protracted l.p. differed in essence from the changes of ordinary l.p. (Fig. 4). In intervals up to 10 msec the protracted l.p.s overlayed one another; a strengthening of the second potential was never seen, and indeed it was always weakened. Where the intervals were larger the second protracted l.p. disappeared altogether. A gradual

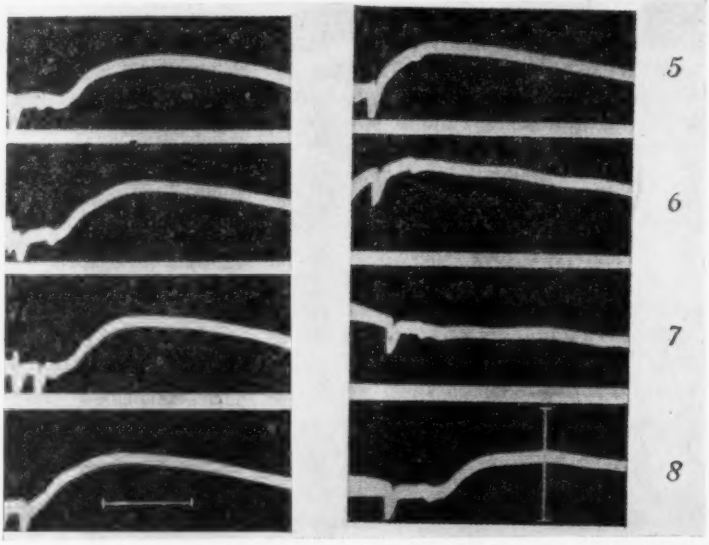

FIG. 4. Prolonged local potentials. (1) one stimulus. (2)–(8) two stimuli, with intervals of 2·2, 4·5, 6·7, 11·2, 15, 45, 75 msec. Scale: 30 mV, 20 msec.

re-establishment of the second protracted l.p. occurred only with intervals of more than 50 msec and a full re-establishment occurred only with intervals of a few tenths of a second. At the period when protracted l.p. overlayed one another, no conversion to p.p. was ever observed.

Such l.p.s greatly resemble the effects of tonic fibres described by Kuffler and Williams [18] as "small nerve-junctional potentials". However, we must mention that these protracted l.p.s were registered by us in the fibres of the sartorius muscle, where the presence of tonic components is denied.

Rhythmic stimulation. Peak potentials both from rhythmic stimuli and double stimuli differed a little according to how far from the origin of the waves of stimulation the intracellular microelectrode was introduced. If the p.p. were recorded a few millimetres from either the end plate or the place of direct stimulation then with frequent stimuli either all p.p. except the first were visibly weaker (Fig. 5), or the p.p. alternated in amplitude (Fig 5 *2*). A clearly noticeable weakening of p.p. started at a frequency of 80 c/s and then gradually and proportionately lessened as tetanization continued.

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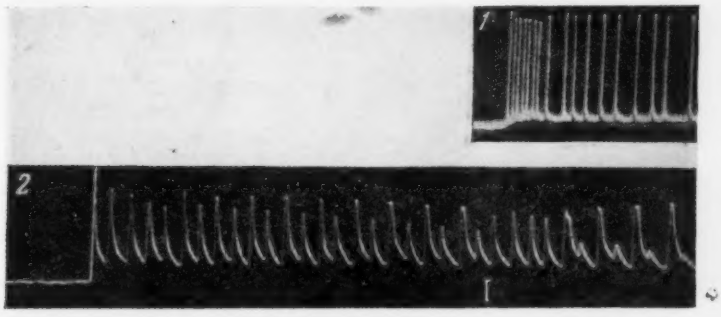

FIG 5. (1) peak potentials of the muscle fibre with indirect stimulation at a frequency of 250 c/s. (2) same in another fibre with a frequency of 150 c/s; Recording in the myoneural area, high rate of development. Scale: 25 mV.

If the recording of potentials was taken at a considerable distance from the origin of the waves of excitement we were unable to observe the p.p. which had been weakened by the relative refraction after preceding impulses. From the start, where the frequency of excitation was high the rhythm of the p.p. was transformed and the amplitude of the p.p. was a maximum. Obviously the reason for this is the delay in the spread of weakened impulses associated either with their escaping from the zone of refractoriness and simultaneous re-establishment to the maximum size, or with their total extinction.

As for the transformation of the rhythm seen when the microelectrode was placed near the origin of the impulse, with indirect excitation this can be explained by the changes occurring in the synaptic area itself. But transformation also occurred in direct excitation. Evidently when frequent waves of excitation occurred in the muscle fibre a fairly rapidly progressive delay in onset of individual impulses occurs; this involved a lowering of lability and led to a corresponding protraction of the periods

of refractoriness. It was precisely on such occasions that no rapid change from one rhythm of discharge to another occurred in the muscle fibres, but a gradual evolution of alternating rhythm with a distinct lengthening of the individual p.p. especially in their descending slope was seen.

In frequencies of over 50 c/s a certain interaction between l.p. with rhythmic excitations was observed. Successive l.p. overlayed each other and generated a stable depolarization of the membrane of the end plate, on the background of which

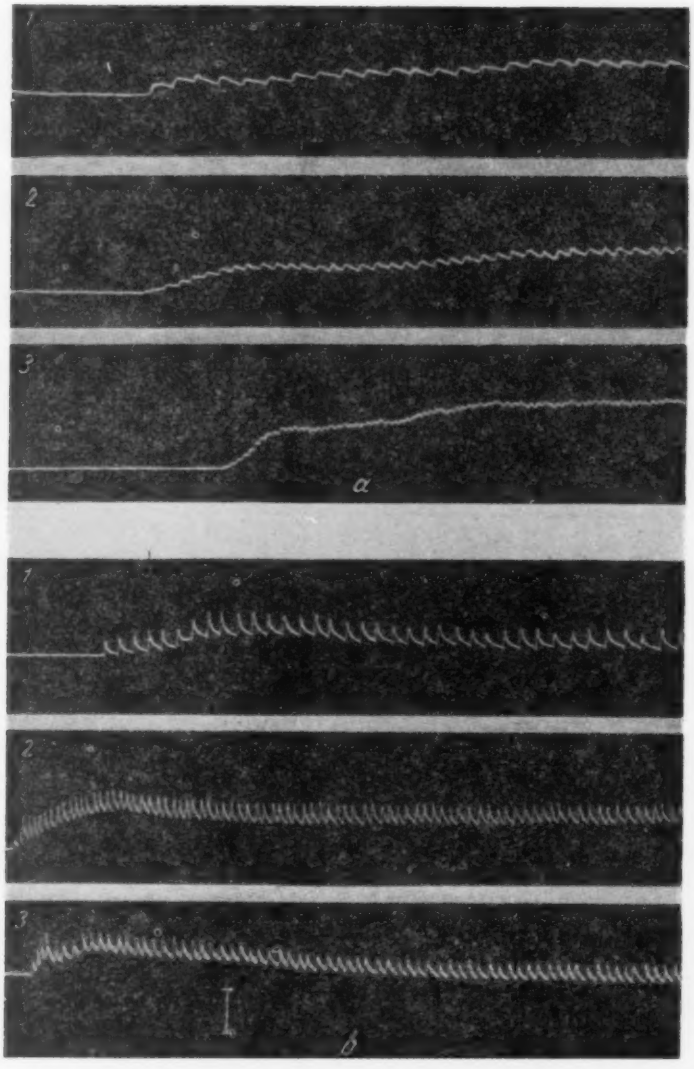

FIG. 6. Local potentials of the end plate with rhythmic indirect stimuli. (a) deep curarization, (1)–(2)–(3) frequency of stimulation respectively 50–70–200 c/s. (b) Light curarization, (1)–(2)–(3) respectively 50–100–200 c/s. Scale: 25 mV.

could be seen individual waves of potential in the rhythm of excitation—this has been mentioned already by a number of authors [19, 20, 6, 8, 9, 11]. Usually the stable depolarization of the end plate increased throughout the first 10–20 impulses. The increase was more rapid when the excitation was most frequent. A plateau occurred, leading to a slowly descending slope. Finally all stable depolarization disappeared in spite of a continued excitation. Its intensity did not exceed 20–30 mV at the maximum period. Complete disappearance of individual waves never occurred. When Ginetsinskii and Mikhel'son [19] observed the disappearance of rhythmic oscillations it was probably connected with some inertia in the recording apparatus.

The changes of individual l.p. arising in the rhythm of excitation against a background of stable depolarization, and the ratios of their intensity to the intensity of the latter, were different with different preparations. With deep curarization transmission in all fibres was fully removed and a particularly marked stable depolarization accompanied the rhythmic stimulation (Fig. 6a). Individual local potentials changed relatively little along the path of excitation. Under these conditions the correspondence between frequency and rhythm of stimulation was a characteristic peculiarity of the l.p. No signs of transformation of the rhythm or fall of individual l.p. were observed up to and over 200 c/s (Fig. 6 *3*).

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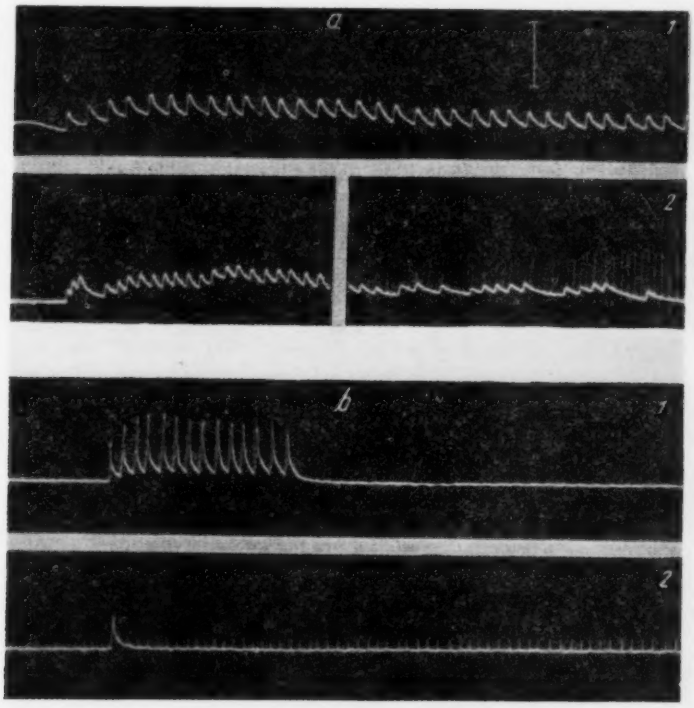

FIG. 7. Local potential of the end plate with rhythmic indirect stimuli without curarization. (a) Frequency of stimulation 50(1) and 150 c/s (2). (b) Frequency of stimulation 50(1) and 100 c/s (2). Scale: 25 mV.

With less deep curization individual l.p. get stronger during the period of growth of a stable depolarization; the ratio of magnitudes of individual waves to the stable depolarization increased (cf. the oscillograms, Fig. 6a, b). Transformation of the rhythm also occurred more easily. The end plate responded to the frequency of impulses at 200 c/s with even fewer variations of the potential than with a frequency of 100 c/s.

Sometimes we succeeded in recording the l.p. of the end plate without curarization. On these occasions the stable depolarization was even weaker, with intense individual l.p., and transformation of the rhythm of the l.p. with increased frequency of excitation occurred quicker. Usually a half rhythm was observed when the frequency of excitation was 150 c/s (Fig. 7a). Also, after short intervals of time, whole groups of l.p. had entirely fallen out. Eventually all changes of the membrane potential of the end plate ceased.

An extreme degree of transformation of the impulse frequency in the end plate occurred on occasions when the plate responding to a lower frequency with a series of l.p., gave only an individual l.p. when the frequency had been increased up to 100 c/s in response to the first motor impulse (Fig. 7b). When recording from nerveless parts of the muscles, we could find fibres which, with a relatively small frequency of excitation, gave only one p.p. in response to the first nerve impulse.

The phenomena of transformation of the rhythm of the l.p. of the end plate, described above, are doubtless associated with processes which develop in the synaptic area, and not in the nerve fibres. Recordings of action currents of the sartorius muscle during such stimulation showed that, with a frequency of 150–200. c/s, when transformation of the l.p. rhythm developed, the action currents of the nerve remained unchanged both in amplitude and in frequency.

SUMMARY

The observations described show how complicated are the processes involved when rhythmic impulses are conveyed from nerve to muscle and when they are conducted further along the muscle fibre. A relatively low rate of conduction of a stimulus along the muscle fibres offers favourable conditions for showing the results of delayed movement of impulses weakened by refraction. The weakened impulses faded out. The stronger ones, even at a distance of 0·5–1 cm, managed to escape from the zone of refractoriness and to re-establish themselves up to their maximum size. Such a peculiarity of stimulation is responsible for the fact that only the discharge of the maximum impulses, in most of the muscle fibre, were observed, and then with a frequency not higher than 80 c/s. The excitation rhythm mentioned above was fully adequate to sustain a steady tetanic contraction of the muscle fibre. Therefore we must consider that the appearance next to the end plate of impulses weakened by relative refraction, with frequent stimuli, which exert a depressing effect, could not be the real reason for a corresponding weakening of the muscle counteraction.

The rhythmic activity of muscles was also largely determined by the properties of the synaptic junction. Two types could be discerned: a pre-synaptic terminal ramification of the motor nerve fibre and the end plate which is generally accepted as a

post-synaptic formation, and as a specialized part of the muscle fibre. Intracellular recording is only possible from the second type: a convenient method of isolated study of its activity is the recording of potentials with curarization.

However, with curarization, as our findings show, the course of rhythmic processes in the end plate differs from their normal course. With curarization the end plate produced greater frequencies in its l.p. than without it. This conclusion is not new: Göpfert and Schaefer [21] have shown that the transformation of rhythm and depression inhibition are much better expressed without curarization than with a partially curarized end plate The non-curarized myoneural junction is the link whose activity can be most easily disrupted by rhythmic excitation Its activity can be considered, and has been put forward by Vvedenskii as the least labile part of the neuro-muscular preparation.

It was noticeable with mild curarization that the transformation of the l.p. rhythm, the emergence of intermittent l.p., and finally their total disappearance, were not connected with a parallel increase of stable depolarization of the end plate. The final result of depressive stimulation was a total disappearance of all changes in the membrane potential of the end plate. It makes us suppose that, contrary to a widespread opinion, the depressive condition in non-eserinized myoneural junctions is not a direct result of stable depolarization of the end plate under the effects of frequent nerve impulses. It is probable that with depressive inhibition all activity of the nerve ending at the end plate may cease. (Ginetsinskii and Mikhel'son [22] admitted this when they originally studied the changes of electrical reactions of the myoneural area in depression in detail.)

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DISCUSSION

(1) With the help of intracellular recording the potentials of various parts of muscle fibres were studied; the sartorius muscles of frogs were used, applying double or rhythmic direct or indirect stimulation.

(2) If peak potentials were recorded from tissues a few millimetres from the myoneural area with indirect stimulation or from the site of direct stimulation, then when using double stimuli an absolute refractoriness of 3·7–5·0 msec and a relative one of 10–20 msec could be demonstrated. When recording peak potentials far from the point of excitation in the fibres it was not possible to determine the duration of relative refractoriness. Impulses weakened by relative refractoriness spread very slowly. While doing this the greatly weakened impulses faded out completely and the less severely weakened ones escaped from the zone of relative refraction and were restored to their maximum size.

(3) In recordings from the end plate taken on curarized and sometimes also on the normal muscles using indirect stimuli, local potentials were registered which summated when the intervals between stimuli was 2·5 to 15–20 msec; with mild curarization the second of these became stronger in comparison with its original magnitude. With large intervals of 75–100 msec the second potential was somewhat weakened. If local potentials arose in the muscle fibre itself, as was the case in conduction disturbances, they were also connected with the capacity for summation.

(4) With rhythmic stimulation of muscle fibres with a frequency of 80 c/s and higher, peak potentials corresponding to the frequency of excitation were recorded

only close to the origin of the impulses, although weakened or alternating in amplitude. When recording was taken further away, only discharge from it of maximum peaks of a frequency less than 80 c/s was observed. With the same stimulus in the area of the end plate in the case of deep curarization, local potentials were recorded which coincided with a rhythm of stimulation up to 200 c/s. These potentials were superimposed on an intense protracted depolarization. Without curarization or with mild curarization the stable depolarization of the end plate was found to be weaker, and transformation of the rhythm of separate local potentials occurred with less frequency. Alternating rhythms of local potentials or complete disappearance of them occurred easily in spite of the continued stimulus of the nerve.

(5) It is presumed that depressive inhibition with frequent stimuli is not directly connected with protracted depolarization of the end plate.

Translated by E. L. URCH

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THE INFLUENCE OF THE STRENGTH OF STIMULATION ON THE MAGNITUDE OF LOCAL ELECTRIC REACTION IN A NERVE*

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THE "gradual" theory of stimulation [1–6] has recently been described in articles by Nasonov and his colleagues. The basic tenet of this theory is that the reaction of the stimulated fibre shows a gradual dependence on the strength of the irritating stimulus and the curve representing this dependence is S-shaped (Fig. 1c). Starting with this the "gradual" theory establishes the laws of the relationship between local and propagated stimulation. According to the theory, the constancy of magnitude of the action potential is the result of its unique self-regulation in passing along the fibre. Depending upon the strength of the stimulating impulse, both increment and decrement conduction can be observed near the point of stimulation. Therefore study of the local reaction is of special importance, because the position of an S-curve in a co-ordinate system, where the strength of the stimulus is graphed along the abscissa and the magnitude of the responding reaction along the ordinate, should not only characterize the functional condition of the fibres at the point of excitation but also indicate how well maintained is the conduction of the nerve impulse along the fibre. The basic circumstances and conclusions of the gradual theory are confirmed both by experiments which were set out to check the theory and by facts well known in literature on the subject.

FIG. 1. Different forms of dependence of the magnitude of the local reaction on the degree of excitation. Explanations to be found in the text.

The following facts also apply here:

(1) The appearance of a sub-threshold local response propagated with a decrement [7–9].

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† It is necessary also to consider Herman's theory of "small currents" which allow conduction of an impulse along a nerve fibre.

(2) The presence of an incremental build-up of the stimulation when the threshold is reached [8].

(3) The possibility of decremental propagation in response to a powerful stimulus [4, 10].

(4) The increase of threshold local response and decrease of the action potential when there is a decline of excitability reflected by a drop in the so-called "safety ratio" [8, 10, 11].

However, although the Nasonov "gradual" theory can be considered sufficiently well founded, a complete S-shaped dependence of the magnitude reactions on the strength of the stimulus has not been obtained by anyone up to now. Moreover, almost all facts which confirm the gradual theory have been obtained on isolated non-medullated nerve fibres, while a number of authors describe a dependence of the "all or none" type (Fig. 1a) [12] in the myelinated fibres of the frog in local reaction. This author considers that these findings do not coincide with the facts indicating the possibility of a gradual dependence on the strength of the stimuli as found by Hodgkin [8] (Fig. 1b) because of the difference in structure of myelinated and non-myelinated fibres.

It is interesting to study the dependence of local reactions on the strength of stimulation in the nerve of a frog, in order to determine (1) how possible are gradual relationships between the strength of a stimulus and the magnitude of the reaction for nerves composed of myelinated fibres, and (2) the possibility of studying the form of this dependence more fully.

METHODS

The object and equipment used. We used the sciatic nerve of the frog for our research. Stimulation of the nerve was by rectangular pulses of current from an impulse-generator with an internal resistance of more than 3 MΩ. The time of growth and decline of pulses did not exceed 5 μsec. Their duration varied from 20 μsec to 4 msec. The stimulating pulse could be released at any place along the line of its growth by means of a special method of starting of the generator by the voltage on the oscillograph. This made observation of the first moments of excitation convenient. The amplitude of the pulses of current at the output terminal of the generator could be changed smoothly from 0 to 12 μA and from 0 to 60 μA, and the strength of the current set with a precision of 0·2 μA and 1 μA respectively. The frequency of stimulation was measured by the frequency of repetition of pulse build-up and could change from 5 to 500 c/s. We also used single excitation. Most tests were conducted with a frequency of excitation of 25 c/s. In the control series we used single stimuli. Potentials led off from the nerve were applied to the plates of the oscilloscope for vertical displacement of the electron beam through an amplifier with a differential input terminal. The coefficient of magnification of the amplifier ensured a maximum sensitivity of 400 μV/cm. The ratio of the cophasal signal to the antiphasal (when the deflection was the same at the outlet) exceeded 3000. The time of establishing the signal at the output terminal of the amplifier when a rectangular jump of voltage was given at the input terminal did not exceed 0·4 μsec. The input resistance between earth and each input terminal of the amplifier was 2 MΩ.

Recording of potentials was obtained by cinephotography and simultaneous visual observation of the oscillogram using two oscilloscopes.

Compensation for the electronic potential. Study of the local electric reaction on a whole nerve trunk meets with considerable difficulties, due to the presence of a so-called electrotonic potential which gets recorded at the point of the stimulation and entirely conceals the local reaction of the nerve. This makes it absolutely necessary to balance the electrotonic potential. We used the special method which was described in detail in a previous article [13].

Unit and electrodes. We used the unit described by Shmitt and Shmitt [10] in a slightly modified form. In this unit the electrodes were mounted on hinges in the sides of the case, and could move freely in any direction, convenient for placing electrodes on a nerve. In order to be able to study the spread of the action potential as well as the local response we fixed 5 electrodes firmly on a nerve. With the help of a switch we were able to make various recording circuits, shown in Fig. 2. In Fig. 2a is given the circuit for recording the local response initiated from electrode 3 with the use of compensating voltage applied to the second input of the amplifier. In Fig. 2b and c are various circuits for recording the action potential when the compensating circuit is cut off. Electrode 3 is non-polarized; the other electrodes are silver.

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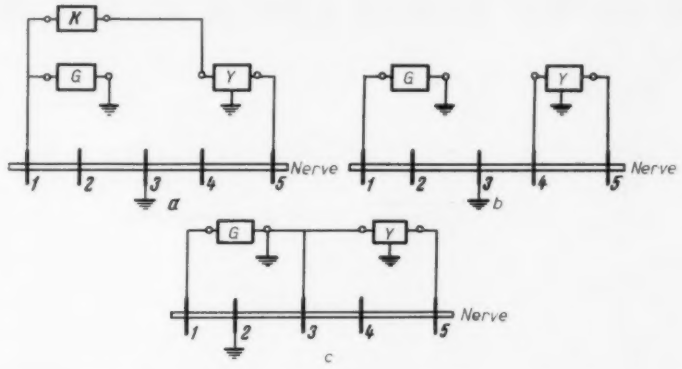


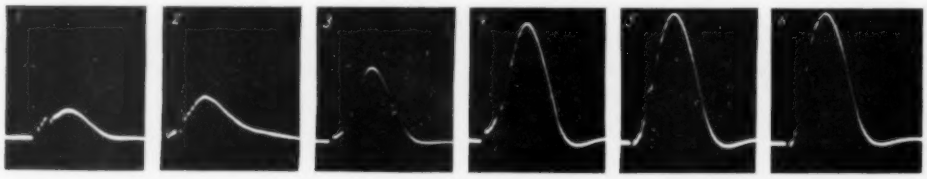
FIG. 2. Recording scheme of the local response and of the action potentials in the nerve. *G*—Generator of rectangular pulses. *Y*—Amplifier with a differential input terminal. *K*—Generator of compensating voltage.
(a) Diagram of recording of local responses of the nerve. (b) and (c)—Diagrams of recording of action potentials.

RESULTS

In the first series of tests the dependence of local reaction on the strength of the stimulus of 300 μsec duration was studied. For this we recorded the local responses occurring at electrode 3 with stepwise increases of stimulation. The results are given in Fig. 3. In Fig. 3b curve *1* shows the dependence of local response on the strength of the stimulus, and is S-shaped. We know that the curve of action potential on the strength of the stimulation of the intact nerve has the same form, with the attainment of a constant value when all the fibres are active. The question arises of

how much the S-shape of a local response reflects a true dependence of each nerve fibre and how far it is dependent on the statistical curve obtained as a result of gradual activation of the individual fibres. To solve this we compared the curve of a local response with two other curves obtained by recording potentials of action far from the stimulating electrodes.

Curve *2* (Fig. 3b) was obtained by recording the action potential which occurred at the same electrode (3) as the local response, but was recorded far from it (4). There are two interesting points about this curve: (1) the beginning of the curve characterizing the threshold of conduction, i.e. that strength of stimulation at which the local response at electrode 3 begins to be propagated; and (2) the reaching of a steady value in the curve, corresponding to the strength of stimulation of the least sensitive fibres, at which strength all fibres begin to conduct. A comparison of the absolute values of curves *2* and *1* is impossible since they are obtained from recordings of the potential at different parts of the nerve, i.e. under different conditions (such



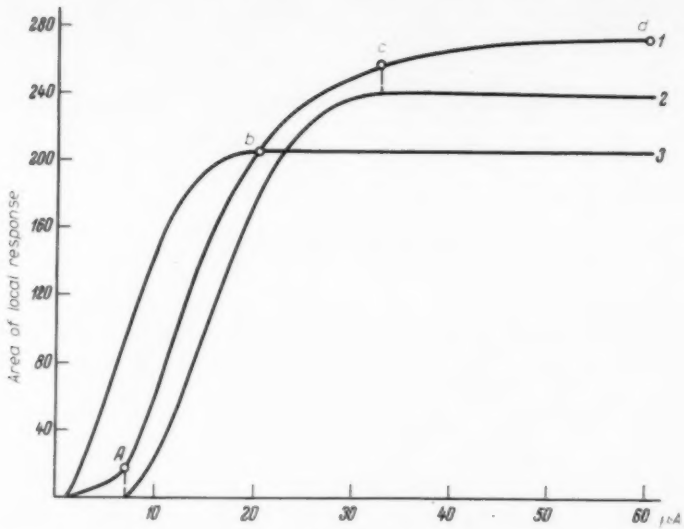


FIG. 3. Curve of dependence of local response of the nerve upon the strength of stimulation. (a) Oscillograms of the local response of the nerve (experiment No. 115 done 22 March 1957). Strength of the stimulation (in μA): (1) 14·5; (2) 20; (3) 30; (4) 40; (5) 50; and (6) 60. Threshold of conduction 20 μA; the amplitude in (3) has been reduced 2·5 times. (b) Curves of local response (1) and the action potential (2) and (3) as dependent on strength of stimulation (taking an average of 20 tests). Explanations to be found in the text.

as the thickness of the nerve, the quantity of fibres involved, inter-electrode distances, etc.).

Curve *3* (Fig. 3b) is obtained by recording the action potential, which occurs in another place (under electrode 2) but is recorded by the same electrodes as the local response (electrode 3 and 5). The absolute value of the running action potential with maximum stimulation is the only item of interest on it to us. The beginning of this curve cannot be used for comparison as it concerns a different site of stimulation under different conditions, such as excitability and thickness of the nerve.

Comparison of a curve of the local response (*1*, Fig. 3b) with curves of action potential (*2* and *3*, Fig. 3b) reveals in the following:

(1) The portion of the curve of local response below the threshold of conduction for the most sensitive fibre (that is, below point *A* on curve *1*, Fig. 3b) characterizes an area of sub-threshold local response of the nerve, conducted with a decrement over a short distance. In Fig. 3a, we give an oscillogram of the sub-threshold local response. With respect to duration the sub-threshold local response did not differ from the response above the threshold which spread along the fibre. The dependence of the sub-threshold local response on the strength of stimulation exhibited a convexity in the direction of the abscissa. Considering that an isolated fibre gave a curve of the same form, it may be thought that in the nerve we were dealing with a genuine gradual dependence not complicated by statistical mechanisms.

(2) In tracing the threshold for the least sensitive fibres (Fig. 3b, point *C*) on the curve we see that it continued to rise, although all the fibres had been activated. The portion of the curve lying above the threshold of the least sensitive fibre expresses a gradual dependence on which the statistical factor of separate fibres being activated exerts no influence.

(3) Comparison of the magnitude of the maximum action potential (Fig. 3b, point *C*) with that of the maximum local response (Fig. 3b, point *D*) shows that the local response, in an average of 19 tests, exceeded the action potential by 25 $\pm$ 3·9 per cent which allows these findings to be considered statistically valid.

Under these conditions the difference between potentials which were compared is that one of them originated at the recording site, the other came from further away, and in both cases all the fibres were in action. These facts fully confirm the findings of Averbakh and Nasonov on the presence of super-threshold conduction with a decrement of the local response up to a steady value.

(4) As seen from Fig. 3b, point *C* of the curve of local response corresponding to the threshold of the least sensitive fibre and point *B* of the curve which is equal to the maximum running action potential and recorded at the same point of the nerve, do not coincide. The maximum action potential was not only less than the maximum local response as shown above, but also less than the local response corresponding to the point of activation of the last fibre.

This disparity between local and running stimulation at the threshold of stimulation for the least excitable fibre cannot be explained by the rule of "all or nothing" by which each elementary local reaction must be equal to the elementary running potential of action. This fact becomes understandable only if we postulate that the local reaction, equal to the maximum action potential, contains greater responses of more sensitive fibres which are conducted with a decrement, responses

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equal in size to the running stimulation, and small responses of the less excitable fibres which are conducted with an increment. Their summation may be equal to the maximum action potential when part of the fibres have not yet been activated. When the threshold of the last fibre is reached the largest local responses must predominate, spreading with a decrement, so that the total local potential is considerably higher than the spreading action potential.

Increase in stimulation leads to further growth of local responses in individually less sensitive fibres. This is expressed by a continued rising of the curve. In this way the excess of local response over the action potential at the threshold of stimulation for the least sensitive fibre also shows the gradual dependence of local response on the strength of stimulation and on the presence of decrement of excitation.

On the basis of our detailed analysis of various sections of the S-shaped curve of local response by comparing it with the curves of action potential, one can deduce that a statistical mechanism, inherent in many curves, by no means exhausts all dependencies, and, consequently, the S-shape of the curve of local response is a true law for each individual fibre.

The aim of the following stage of our work was to study the curve of local response to the action of stronger stimuli. For this, in the second series of tests, we used stimuli of 3 msec duration.

Study of the curve of local response as dependent on the strength of a stimulus of 3 msec duration showed us that with powerful stimuli, which exceeded the threshold of conduction about 10 times, the curves of local response begin to decline toward the abscissa, i.e. a stronger stimulus provokes a lesser response than the weaker one (Fig. 4). This phenomenon is not connected with damage to the tissues with powerful current since it is easily reversible. The curve can be reproduced in both directions, with the same effect. It is also not connected with the action of repeated excitations, since individual stimuli used in the control series give the same reaction. Consequently the drop in the curve can be considered due solely to the

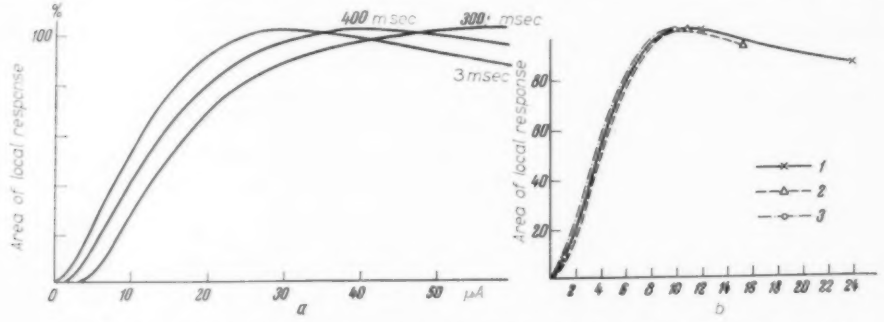

FIG. 4. Curve of dependence of local response on strength of stimulation with varied duration of the stimulus. (a) Strength of stimulus in μA along the abscissa; the square of the local response as a percentage along the ordinate, where the square of maximum local response is taken as 100 per cent. (b) The strength of stimulus at the threshold of conduction along the abscissa; the square of local response as a percentage along the ordinate. The square of maximum local response is taken as 100 per cent. (1) duration of stimulus 3 msec; (2) duration of stimulus 400 μsec; (3) duration of stimulus 300 μsec.

strength of the stimulus and we evidently were dealing here with the phenomena of optimum and pessimum of the strength of the stimulus in the pure form.

Comparison of the curves of local response upon varying the duration of the stimulus shows no noticeable influence of stimulus duration on the curve.

Actually, the curves of differing duration of stimulation, 3 msec, 400 μsec and 300 μsec are different (Fig. 4a). The most favourable value for short periods is more to the right, i.e. in the region of the stronger stimuli. However, such a comparison of the curves is invalid, since it does not allow for the dependence of the strength on the duration of the stimulus. If the strength of the stimulus is calculated not in absolute units but in thresholds (Fig. 4b) and the reactions in percentages of the maximum, then the curves for various durations lie one upon the other their optima coincide, and the varying degree of drop of the curve is explained by the inadequacy of the strength of excitation under our conditions. One may postulate that the stronger stimuli prolonged for 300–400 μsec would cause a similar drop in the curve. The average results in this series of tests are given in the Table 1.

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The size of the maximum local reaction, i.e. the response to a stimulus of optimum strength, is different for differing durations of stimulation. On an average from 19 tests, in each of which we compared the square of maximum local response to a stimulus of 300 μsec and of 3 msec, the maximum local response at 3 msec is 87 ± 3·1 per cent of the response at 300 μsec and the spike is 80 ± 3·9 per cent of the spike at 300 μsec. The difference between the responses to different durations of stimulation was verified statistically.

TABLE 1. CHANGE OF THE FORM OF THE CURVE OF LOCAL RESPONSE AS DEPENDENT ON STRENGTH OF STIMULATION OF VARYING DURATIONS

Duration of stimulus (μsec)	Number of tests	Threshold of conduction (μA)	Optimum stimulation in thresholds	Maximum excitation (60 μA) in thresholds	Square of local response to the optimum stimulus (%)	Square of local response to the maximum stimulus (60 μA) (%)
4000	62	2·0	12	24	100	86 ± 1·4
400	19	3·9	11	15·4	100	93·5 ± 2·0
300	30	6·0	—	10	100	100

The results of an additional series of 9 tests where we compared the curves of the local response to stimulation over a wide range of durations of 100, 200, 300, 400 μsec and 1, 2, 3, and 4 msec, showed that the optimum duration of the stimulus for a maximum local response lies in the region of physiological durations of 300–400 μsec. But these results are only preliminary.

DISCUSSION

Analysis of the dependence of local response on the strength of the stimulus in the intact nerve trunk shows that this dependence cannot be wholly conditioned by

the statistical distribution curve of thresholds, which is confirmed by the following facts.

(1) The local response to sub-threshold stimulation of the nerve discloses a gradual dependence on the strength of the stimulus, which is a reflection of true dependence of each individual fibre.

(2) The local response to a stimulus which corresponds to the threshold for the less sensitive fibre exceeds the maximum spike by 20–25 per cent which indicates predominance, in the total local response, of potentials which spread with a decrement.

(3) Further increase in stimulus causes a further rise and then a drop in the curve, although all the fibres have been activated. The portion of the curve above the threshold for the least sensitive fibre shows a genuine gradual reaction upon which the statistical distribution of thresholds has no influence. Our results fully correspond to those of Katz [14] on the sub-threshold local response of the intact nerve of the frog, and to the results of Averbakh and Nasonov [4] on the presence of a sub-threshold decremental spread of stimuli.

As for the form of the curve for the dependence of local reactions on the strength of stimulation, we can now conclude that the curve must pass through a maximum corresponding to the optimum strength of the stimulus and then drop to zero (Fig. 1d). Indirect facts supporting this point of view can be found in the work of Bishop [15].

The S-shaped curve postulated by the "gradual" theory of Nasonov is evidently only the initial half of the whole function. The rule of self-regulation of a running stimulus not only remains in effect but is confirmed by new facts. It is known that the local reaction to an applied stimulus serves as the stimulus to the neighbouring parts of the fibre. When the reactions to depressingly strong stimuli occur, these reactions as determined by the curve described above, will always be less than the stimulus causing them. Consequently, upon stimulation of the neighbouring parts they, being weaker than the stimulant, will be on the initial half of the curve of dependence, i.e., in the region of the S-shaped curve and self-regulation of the running stimulus consequently occurs. The position would only change if the stimulus were so strong that the local reaction were sub-threshold when stimulating the nearby portions of the fibre. Propagation without decrement should then not exist. The existence of a second threshold of conduction should be confirmed experimentally later.*

The findings allow us to conclude that the dependence of local reactions of a whole nerve trunk of a frog on the strength of the stimulus fully complies with the requirements of the "gradual" theory of excitation put forward by Nasonov.

SUMMARY

(1) The local electric reaction of a nerve trunk of the frog is in gradual dependence on the strength of the irritating stimulus. This cannot be fully due to the statistical distribution curve for thresholds.

(2) The gradual dependence of the local response on the strength of the stimulus is expressed: (a) in a heightened local response which corresponds to the threshold

* Under our conditions the strength of stimulus never reached the second threshold, and with any degree of local response the size of the action potential remained constant.

of the least sensitive fibre to the maximum spike recorded at the same part of the nerve; (b) as the stimulus gets stronger than the threshold of the least sensitive fibre by a continued rise in the curve.

(3) The strength of the stimulus is equal to 10–12 thresholds at any duration of the stimulus within the range of 100 μsec–3 msec which are optimal for the local response. Further increase in the stimulus causes a drop in the curve of local responses.

(4) The curve expressing the dependence of the local reaction on the strength of the stimulus must pass through a maximum which corresponds to the optimum strength of stimulus, and then drops to zero. The S-shaped curve postulated by Nasonov's "gradual" theory is the initial half of the whole function.

(5) The results show that local reaction of the nerve trunk of the frog follows the rules of Nasonov's "gradual" theory of excitation completely.

Translated by E. L. URCH

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THE ROLE OF REDOX POTENTIAL CHANGES IN PLANT REGENERATION PROCESSES*

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THE experimental data on the effects of an external e.m.f. on regeneration in tomatoes were given in our previous paper [1], where it was shown that an external e.m.f. of appropriate direction and magnitude could greatly accelerate the formation of the callus and of accessory growing points. The potential difference (p.d.) and electrophysiological polarization in the tissue greatly influence the migration rates of materials [1–10]. Work is required to bring up or remove material in a tissue, and the second law of thermodynamics indicates that this can only occur if there is a free energy gradient. The gradients within tissues are increased by the external e.m.f. and cytological reactions thereby stimulated. As the redox potential reflects the energetics of metabolism in a way similar to the bioelectric potential, it may be that regeneration could be accelerated or retarded by altering the Eh in the protoplasts.

The methods of producing calluses and of measuring bioelectric potentials were the same as were used in [1]; the redox potential was determined potentiometrically, the indifferent electrode being of platinum and the reference electrode being the calomel one. A Weston standard cell was used, the null detector being a GMP-1951 galvanometer. Special vessels and a nitrogen atmosphere were used in determining redox potentials; pH was determined by the quinhydrone method. Smoll's calorimetric method for pH, and Clark's method for Eh were also often used; each series of experiments was repeated not less than 10–15 times. The redox potentials of stem tissues were altered by injecting oxidants or reductants, the main oxidant used being 0·1% H_2O_2 and the main reductant 0·01% hydroquinone. These concentrations were such as not to result in direct damage. These agents were chosen as being those used in vegetative plant reproduction.

REDOX POTENTIAL CHANGES DURING CALLUS FORMATION

Calluses began to form on the stem tips and peduncles on about the twenty-fifth day after operation, Eh and rH_2 increasing considerably at this point (conditions more highly oxidizing—Fig. 1). The difference in Eh between the parenchymal cells of the callus and of the original stem varied from +123·5 to +139·0 mV, the difference in rH_2 being 3·3–4·2 units. The young callus cells differed from the stem in having a somewhat lower pH during life (pH_n), the original stem cells having values of 5·4–5·5, while the young callus had 5·1–5·3. The callus redox potential subsequently increased slightly, the maximum Eh being reached during callus formation being +171 mV in the variety "Shtambovyi Alpat'eva" and +70 mV in

* Biofizika **3:** No. 3, 295–305, 1958 [Reprint Order No. BIO 145].

"Best of All", the corresponding rH_2 values being 16·1 and 12·7. However, these parameters subsequently altered greatly, shortly before traces of the secondary meristem began to appear within the callus; most of the cells began to show a reducing redox potential, with the Eh falling to 115·5–131 mV. The pH also rose to its highest value at this point, becoming 5·7–5·8 instead of 5·1–5·3.

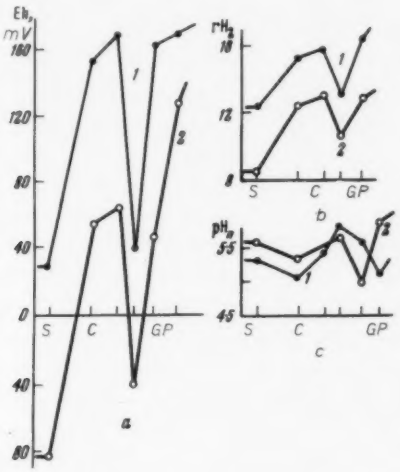

FIG. 1. Eh(*a*), rH_2(*b*) and pH_n(*c*) changes while callus and accessory growth points are forming in "Best of All" (1) and "Shtambovyi Alpat'eva" (2). *S* = stem, C = callus, *GP* = growth point.

The Eh again became high and positive as the secondary meristem began to differentiate, being +169 mV in "Shtambovyi Alpat'eva" and +53 mV in "Best of All", the rH_2 increasing by 3·9 units and 1·5 units respectively, and the pH_n values becoming about 5·1 and 5·0 respectively, when certain colorimetric results are incorporated. Similar changes have been observed in other tomato varieties [11]; in all, four stages in callus formation can be distinguished. In the first Eh and rH_2 are high and pH_n low; in the second Eh and rH_2 are maximal, pH_n being higher; in the third Eh and rH_2 are minimal, pH_n being maximal; while in the fourth Eh and rH_2 are high and pH_n low. In stages one and two the parenchymal cells are developing, while in four the secondary meristem is differentiating, the third falling between these two main phases.

EFFECT OF REDOX POTENTIAL ON REGENERATION IN TOMATOES

Table 1 gives the data from an experiment in which the cut sites on the stems were treated once per day (at 8 p.m.) with H_2O_2 or hydroquinone for 6 days; control cuts were treated with water. The first treatment was given 9 days after cutting, 25 plants being used in each series. By day 15 it had become clear that no calluses had formed in either variety treated with H_2O_2, whereas "Best of All" treated

TABLE 1. EFFECTS OF 0·1% H_2O_2 AND 0·01% HYDROQUINONE ON CALLUS FORMATION IN TOMATOES

Reagent	"Best of All" (%)		"Shtambovyi Alpat'eva" (%)	
	With calluses	Without calluses	With calluses	Without calluses
Water	45	55	46	54
H_2O_2	0	100	0	100
Hydroquinone	56	44	43	57

with hydroquinone showed 12 per cent more calluses than the control. Calluses also formed in "Shtambovyi Alpat'eva", but there was a slight delay in this case.

The Eh, rH_2 and pH_n were determined after the two reagent treatments (Table 2); in "Best of All" the redox potential fell from +114·3 to +74·9 mV after the H_2O treatment, and from +156·2 to 111·1 mV in "Shtambovyi Alpat'eva", the falls in rH_2 being from 15·7 to 14·2 and from 16·5 to 14·9 respectively. The Eh and rH_2 increased when the callus formed, but pH_n fell; the redox potential drops occurring on H_2O_2 treatment suppressed callus formation. In the hydroquinone-treated plants the redox potential rose to 135·4 mV ("Best of All") and 162·1 mV (Shtambovyi Alpat'eva), but in the former pH_n fell by 0·5 unit, while in the latter it rose to 5·99 instead of 5·57. This rise in pH is the cause of the delay in callus formation in "Shtambovyi Alpat'eva".

Table 3 gives the results for day 21 from an experiment in which the cut stems of "Best of All" were treated daily with H_2O_2 or hydroquinone for 20 days, starting on the first day after operation (at 8 p.m.). Here all plants so treated gave calluses which appeared much earlier with the hydroquinone treatment, but while 14 per cent of the plants gave large calluses with H_2O_2, the rest gave none at all. The hydroquinone treatment produced a persistent shift in the positive potential to the higher- and lower-lying cells (Table 4), the p.d. in all cases being large compared with the control (electrodes 15 cm apart).

TABLE 2. EFFECTS OF 0·1% H_2O_2 AND 0·01% HYDROQUINONE ON Eh, rH_2 AND pH_n WHEN THE CUT STEM SURFACES ARE TREATED

Reagent	"Best of All"			"Shtambovyi Alpat'eva"		
	Eh (mV)	rH_2	pH_n	Eh (mV)	rH_2	pH_n
Water	+114·3± 1·7	15·67±0·12	5·87±0·04	+156·2±17	16·52±0·07	5·57±0·09
H_2O_2	+ 74·9± 1·1	14·18±0·03	5·80±0·06	+111·1± 8	14·91±0·05	5·54±0·03
Hydroquinone	+135·4±10·1	15·52±0·07	5·43±0·02	+162·1±13	17·56±0·14	5·99±0·16

Leaf petioles were used as the upper points, the hydroquinone being applied to the cut surfaces of the stems. The H_2O_2 treatment produces a persistent shift in the

negative potential (Table 4), the p.d. being mostly high and negative or small and positive.

In some plants regeneration proceeded normally in spite of the H_2O_2 treatment, and here the potential was not reduced, but became high and positive (Table 4), the tissue polarity being unchanged. In these plants the "buffering" capacity of the redox system was so great that the H_2O_2 was without effect at the concentrations used. The plants in this group were usually well developed in a morphological sense. Normal or accelerated callus formation occurs if the redox and bioelectric potentials are unchanged by H_2O_2 treatment or increased by hydroquinone treatment. Fig. 3 shows how the p.d. is affected by applying 0·1% H_2O_2 to the cut stem surfaces at 17–20 days after operation, the potentials being taken from three points. The solutions were applied very carefully in drops under the middle electrode; the points relate to potentials relative to the cells to which the oxidant was applied.

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TABLE 3. EFFECTS OF 0·1% H_2O_2 AND 0·01% HYDROQUINONE ON CALLUS FORMATION IN "BEST OF ALL"

Reagent	% Calluses		Remarks
	Present	Absent	
Water	100	—	66% young calluses, 34% vestigial
H_2O_2	14	86	No morphological changes at the site of section. Calluses large
Hydroquinone	100	—	54% large calluses, 46% young

The bioelectric potential usually fell immediately to a minimum when the H_2O_2 was applied, any subsequent rise being slight, since continuous observations for 8 hr afterwards indicated a persistent shift. The potential difference relative to lower- and higher-lying stem cells was reduced, and in most cases the polarity was reversed, the H_2O_2-treated areas being electrically negative.

TABLE 4. BIOELECTRIC POTENTIAL CHANGES ON TREATING "BEST OF ALL" TOMATO PLANTS WITH 0·01% HYDROQUINONE AND 0·1% H_2O_2

Method	Water		Hydroquinone		H_2O_2			
	Percentages of plants with identical potentials							
	50	50	5	95	4	94	2	Plants forming calluses
	Plants		Plants		Plants			
a	+21±5	+7±3	+36±4	+21·5±3	+14±5	−9·5±4	−6·5±3	+15·5±3 +32±4
b	+15±4	+22±2	+29±5	+31±6	−12±3	−11·5±7	+11·5±8	+21±2 +9·5±2

NOTE. The mean potential in each method and the maximum deviations are based on 100 measurements, made 24 hr after treatment. The sign of the potential is relative to that of point *A* in Fig. 2.

The potential was increased by applying several drops of 0·01% hydroquinone, and the potential shift was persistent, the potential often remaining high for 8 hr after the application. The potential differences between the stem and the leaf petiole were found to be the greatest if the top leaf was well developed. Fig. 4 shows how the stem potential is affected by applying heteroauxin at 10^{-3} M, 3-indolylacetic acid being used as reductant here, the changes being similar to those found with hydroquinone. Table 5 shows how Eh is affected by heteroauxin, potassium heteroauxin and potassium heteroauxin sulphonate (8 specimens were used in each case). The redox potential was increased by these reagents in the first few days after operation, the largest changes in Eh being found with the last reagent, and the least with straight heteroauxin. The heteroauxins make the cells more reducing, decrease the pH, and increase the ascorbic acid content, when used 20 days after cutting the stem.

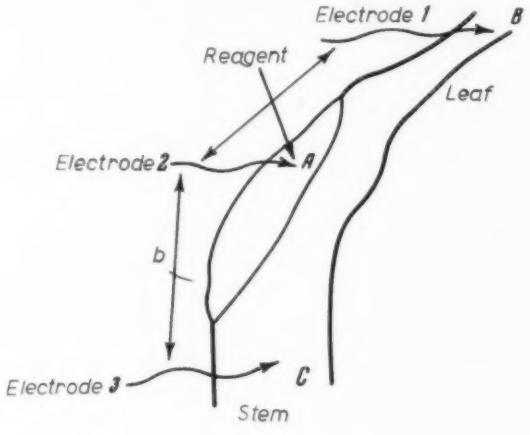

FIG. 2. Placing of electrodes in bioelectric potential measurements.

The rate of callus formation is doubled by the potassium heteroauxin sulphonate, is increased by 66 per cent by the potassium heteroauxin, and 50 per cent by the straight auxin. The growth of the growing points per day was greater than in the control only up to day 15 after treatment, i.e. up to the time where the accessory buds had begun to differentiate. If heteroauxin and its salts were applied to well developed calluses we found no stimulation of regeneration, though the results showed a wide scatter, only 8 out of 50 plants showing acceleration in the appearance of accessory buds. The reduced growth rate in the growing points is here due to the reduced pH and Eh, and to the lower bioelectric potential. The physico-chemical indices of physiological activity are reduced before the secondary meristem begins to form.

Our results show that the bioelectric potential, polarity and redox potential run parallel in our plants when oxidants and reductants are applied; as this subject is a

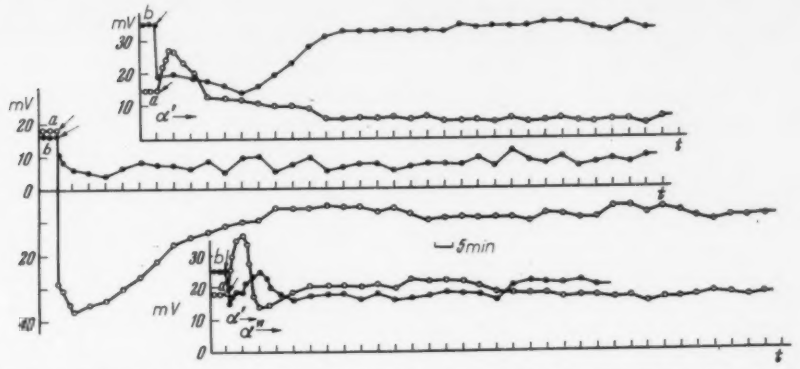

FIG. 3. Potential changes in "Best of All" stems on applying 0·1% H_2H_2. The H_2O_2 was applied at point *A* in Fig. 2, the electrodes being 17 cm apart. (*a*) Potential change relative to tissues lying above, (*b*) the same for tissues lying below. The most typical types of change are shown.

current topic of discussion [10, 14], and is very important in elucidating how protoplasts react to external stimuli, we performed some additional check experiments. When ascorbic acid at 10^{-3} M was applied to the cut surfaces Eh fell from +268·1 to +215·4 mV (Table 5), this corresponding to a reduction in reductant activity by a factor of 1·7; the ascorbic acid content simultaneously fell by a factor of 1·8. It took 5 days for Eh to recover to +285·6 mV, the control value meanwhile being +274·7 mV, corresponding to a 22 per cent rise in reductant concentration, while the ascorbic acid content simultaneously reached the former level, and subsequently rose, as has been demonstrated independently [15].

Use of 10^{-3} M ascorbic acid reduced the potential relative to stem cells lying

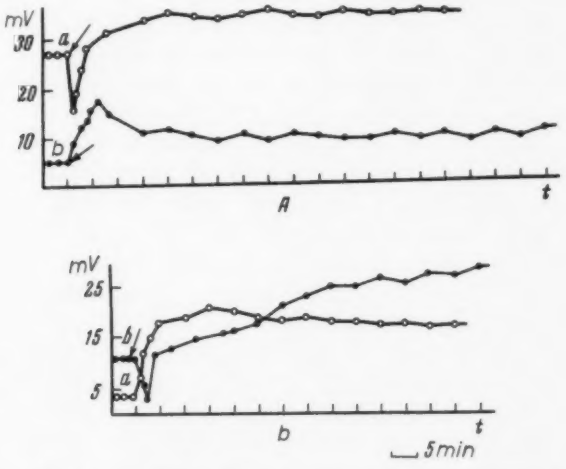

FIG. 4. Potential changes in "Shtambovyi Alpat'eva" stems on applying 3-indolylacetic acid (*A*) and potassium heteroauxin (*b*), both at 100^{-3} M. Distance between electrodes 16 cm. Notation as for Figs. 2 and 3.

higher or lower, the polarity occasionally changing from positive to negative; the value subsequently returned to normal and then increased considerably. Under our conditions the ascorbic acid treatment caused callus formation to cease for several days, only 3 per cent of the plants showing rudimentary calluses 9 days later, while the controls began to produce calluses on the second day.

TABLE 5. EFFECTS OF REAGENTS ON Eh, rH_2 AND pH_n FOR "BEST OF ALL" STEM CELLS

Reagent	First day after treatment			Fifth day after treatment		
	Eh (mV)	rH_2	pH_n	Eh (mV)	rH_2	pH_n
Control (water)	+252·0	20·1	5·7	+274·7	20·7	5·6
Heteroauxin	+276·6	19·9	5·2	+273·9	21·0	5·8
Heteroauxin K salt	+298·7	20·9	5·3	+286·2	21·1	5·6
Heteroauxin sulphonate, K salt	+292·9	21·1	5·5	+308·0	22·0	5·7
Control (water)	+269·9	20·1	5·4	+274·9	20·1	5·3
NaF	+245·4	21·1	6·3	+254·6	21·4	6·3
2:4-Dinitrophenol	+260·7	21·0	6·0	+260·0	21·3	6·2
Control (water)	+268·1	20·8	5·8	+274·7	20·5	5·5
Ascorbic acid	+215·4	17·8	5·2	+285·6	20·4	5·3
NaN^3	+205·6	19·3	6·1	+261·4	20·2	5·6
Malonic acid	+204·9	18·7	5·8	+247·1	19·6	5·5

* All solution 10^{-3} M, except potassium heteroauxin sulphonate, which was $0{\cdot}66 \times 10^{-3}$ M.

At 10^{-3} M NaN_3 inhibits catalase [16], lactase [17] and tyrosinase [18], and reduces the activity of peroxidase by 40 per cent [19], etc.; a single treatment of the cut with NaN_3 reduced Eh from 268·1 to 205·6 mV, pH_n rising from 5·8 to 6·1 (Table 5) and the bioelectrc potential being reduced, becoming negative. This latter change is persistent, the negative polarity lasting for 5–8 days or so. The azide retards callus formation, only 3 per cent of the plants showing callus rudiments at 12 days after treatment, while 39 per cent of the controls had calluses at this time.

Malonic acid inhibits dehydrases, particularly those of β-hydroxybutyric acid [20], of malic acid [21], of succinic acid [22], etc. At certain intracellular pH_n values the malonic acid (10^{-3} M) reduced Eh from 268·1 to 204·9 mV, recovery only to 247·1 mV taking 5 days (Table 5). Callus formation was much delayed, the first signs appearing in 2 per cent of the plants at day 12. The bioelectric potential became negative, and remained so for a long time. At 10^{-3} M NaF inhibits glutamine synthesis [28], phosphyoglucomutase [24], enolase [25–28], etc.; on using it at day 20 after cutting, Eh fell from 269·9 to 245·4 mV, the value being 254·6 mV five days later, while the corresponding control value was 274·9 mV; pH_n also changed from 5·6 to 6·3 (Table 5). Callus formation was again retarded, the first signs appearing in only 4 per cent of the plants after 15 days, while the control showed 48 per cent calluses.

Dinitrophenol uncouples oxidation from phosphorylation [28–30], inhibits aerobic oxidation [18, 24, 31], etc. When the stem tips were treated with it 20 days after cutting Eh fell from 269·9 to 260·7 mV, pH_n increasing by 0·6 unit, this effect continuing to day 5 (Table 5). The bioelectric potential was reduced to very low positive values or even became negative; as before, the effect was persistent. Callus

formation was inhibited, only 3 per cent of the plants showing traces after 15 days, while the control showed 48 per cent calluses.

DISCUSSION

(1) The first conclusion to be drawn is that the changes in cell Eh are unrelated to the Eh of the reagent used, since the cell Eh is not raised by reagents of high Eh, as might be expected, but is reduced; the reverse is found with reagents of low Eh (under our conditions). The cellular ascorbic acid is at first decreased, and not increased, by treatment with ascorbic acid, the pH simultaneously rising and the bioelectric potential falling. The cell ascorbic acid is raised after several days. If high doses are used this is no longer true, but at the concentrations we used it normally applies.

It has often been observed that the intracellular pH increases when plant cells are placed in a medium of low pH, and vice versa [32–34], and it has often been demonstrated [35–38] that reductants do not increase the reducing power but decrease it, while simultaneously increasing the respiration rate, activating peroxidases, inhibiting catalase, etc.; the reactions thus involve activating irreversible oxidation, blocking hydrogen acceptors, and inactivating dehydrase systems. Oxidants at low concentrations mainly activate reduction processes.

The data (together with published results) indicate that the biological reactions to reagents in concentrations which cause no damage are specific [9]; the reactions of plant cells to oxidants and reductants are homeostatic in type and are directed to removing or largely eliminating external disturbances.

(2) The pH and total redox potential changes are not the only ones accompanying changes in callus formation, though they participate actively in formative processes. Redox reactions are a vital part of metabolism, so they may possibly be useful in controlling changes; the reduction in redox potential obtained by treating tomato stems with H_2O_2 retards callus formation. When the redox potential was reduced artificially few calluses appeared in 250–300 plants, and then only in isolated plants where the H_2O_2 concentrations used had no material effect on the protoplast Eh.

"Shtambovyi Alpat'eva" plants always gave calluses when treated with hydroquinone, but they were somewhat late in forming. Here the effect is caused by the increase in pH. Callus formation, and hence production of accessory growth points, was always found in "Best of All" when the cut growing point was treated 20 days later with hydroquinone or heteroauxin. In tomatoes the callus forms when the cells have raised Eh and lowered pH; oxidation inhibitors reduce the redox potential and thus retard callus formation.

The physico-chemical parameters which are responsible for producing the thermodynamic conditions for physiological processes, mainly the redox potential and electrical gradients and polarities, constitute one of the causes of morphogenesis, and are not just metabolic products irrelevant to vital activities.

SUMMARY

(1) The redox potential of tomato stem cells is increased in the first phase of callus formation and reduced in the second, but it again increases when collateral growth points arise and differentiate.

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(2) Oxidants and reductants alter the cell potentials; the callus cells in tomatoes respond to such agents in such a way that their redox potentials alter in the reverse sense to that of the change produced by the reagent.

(3) The bioelectric and redox potential changes produced by a reagent run parallel.

(4) Regeneration in tomatoes is accelerated by applying reductants at low concentrations during callus formation. Callus formation is inhibited by oxidants.

Translated by J. E. S. BRADLEY

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THE EFFECTS OF HETEROAUXIN, HYDROQUINONE AND HYDROGEN PEROXIDE ON THE BIOELECTRIC POTENTIALS OF LEAF TISSUES*

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OUR object in this work was to relate the effects on the organism to the changes in tissue state immediately following application, the substances being chosen for their differing actions on the redox potential (Eh) and on the growth and flowering of the plants. Hydrogen peroxide (an oxidant) usually increases Eh and accelerates flowering somewhat, while hydroquinone and heteroauxin (reductants) have the reverse effect [1]. We used bioelectric potential methods to study the primary changes, the advantage of these methods being that the response is detected directly on the living leaf while still attached to the plant. While Lund's [2] original hypothesis that the biolectric potential was a redox potential has not been confirmed [3, 4], it is undoubtedly true that the redox potential influences the bioelectric one [5]. Whatever may be the origin of bioelectric potentials, their relation to metabolism is not in doubt.

METHODS

Khvedelidze's circuit [6] was used to measure bioelectric potentials, an EO-7 oscillograph being used to measure potential differences. The slowly varying potentials were chopped using a RP-4 relay (Fig. 1a), the sensitivity being 1 mV/mm on the oscilloscope scale. The Ag : AgCl electrode was joined to the leaf surface via a contact (Fig. 1b) so the leaf was not damaged in any way. The solution was applied at the contact area, the concentration being thereby reduced by a factor of 2–3. The potential differences between several pairs of points on the leaf were measured (Fig. 1c) and the absolute change of potential on applying the solution could therefore be calculated. The difference between, say, points A and B is denoted by AB, AB being positive if A is positive relative to B. If AD was measured directly and then calculated as $AB + BC + CD$ the two values did not differ by more than 1–2 mV, i.e. by more than the experimental error. The absolute change of potential on applying the solution could be calculated if one potential difference (e.g. AB, BC, CD, AC, or AD) was unchanged. Only those results in which this was so were used.

No potential differences between solutions of H_2O_2, hydroquinone and heteroauxin as against tap water were observed. Most of the experiments were done on tomato leaves of average age from plants at the initial budding stage.

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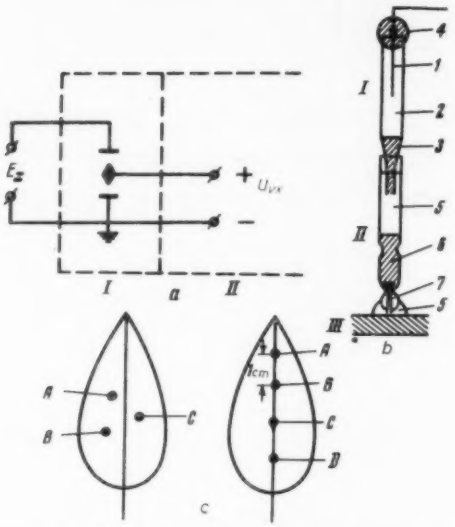

FIG. 1. Circuit for measuring bioelectric potentials. (a) Input circuit; *I*—RP-4 relay; *II*—EO-7 oscilloscope. (b) Electrode and contacts; *I*—electrode; (1) Ag + AgCl; (2) 0·1 N KCl + AgCl sat.; (3) agar + 0·1 N KCl; (4) seal; *II*—contact: (5) tap water; (6) agar + tap water; (7) camel hair; *III*—leaf. (c) Arrangement of electrodes on leaf surface.

RESULTS

Fig. 2 shows the results from one series, in which H_2O_2 and hydroquinone were used; the higher the H_2O_2 concentration the larger the negative shift in the potential, while hydroquinone produced a positive potential (Figs. 2 and 4) which was largest when a 3 per cent solution was used, though this concentration caused leaf burns. Much higher H_2O_2 concentrations were needed to produce burns, so the potentials produced by H_2O_2 are presumably not simply damage potentials but result from some specific action. The H_2O_2 rapidly enters the leaf when applied to the surface and oxygen begins to be liberated; a reddish colour is produced, probably because polyphenols are oxidized.

When tap water or H_2O_2 is applied to a leaf the potential becomes briefly more positive (3–5 mV), but with H_2O_2 the shift soon becomes negative. We found that the tap water and all solutions increased in electrical conductivity after being in contact with the upper surface of an undamaged leaf for 2–5 min, which indicates that some ions must have entered the solution from the leaf. The heteroauxin usually made the potential very slightly more positive at the concentrations we used (Fig. 3), higher concentrations having no different effect; it clearly has no real effect on the processes responsible for the biopotential. Even high heteroauxin concentrations did not produce leaf burns.

The positive potentials produced by hydroquinone have previously been observed on unicellular algae [7]; cresol, phenol, dinitrophenol, benzene, nitrobenzene and hexaresorcinol have similar effects [7–10]. Blinks [7] considers that these compounds affect the potential by interfering with respiration and by altering the ion mobility in the protoplasm. Giant single-celled algae have been used in very careful studies

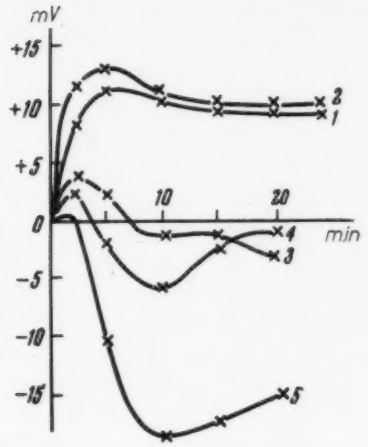

FIG. 2. Potential changes caused by H_2O_2 and hydroquinone. 1—1% hydroquinone (8); 2—3% hydroquinone (6); 3—1% H_2O_2 (8); 4—2% H_2O_2 (3); 5—3% H_2O_2 (4). The figures in brackets are the number of repeats.

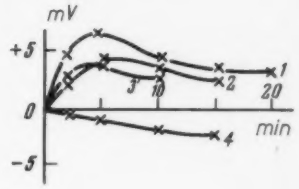

FIG. 3. Potential changes caused by curves 2 and 3 represent different series. The figures in brackets are the number of repeats. Heteroauxin concentrations: 1—20 mg/l (11); 2—100 mg/l (12); 3—100 mg/l (4); 4—300 mg/l (9).

of the metabolic potentials of plants, and it has been concluded that the bioelectric potential results from ion concentration gradients and from the diffusion potential resulting from the alteration in ion mobility at the surface of the protoplasm. The ions mainly responsible for the potential may vary, but K^+ is the most usual one, and it has repeatedly been found that the potential becomes more negative as the concentration of KCl in the surrounding medium increases. The effect arises from the mobility of K^+ being 20–70 times greater than that of Cl^-, and also considerably greater than that of Na^+, the negative shift produced by NaCl being also much less. Hope [11] has found an increased negative potential when KCl acts on roots, the ion mobility relationships being here the same as for single-celled algae. Data on the absorption of ions from solution also confirm the proposed mechanism for the potential; KCl usually behaves as a physiologically acid salt [12], i.e. more K^+ than Cl^- is absorbed.

The potential shifts to the negative side when KCl is applied to a leaf (Fig. 4), the maximum negative potential and the time course of the curve being as for unicellular algae [13–15]. The rise in the first 5 min indicates that the effect is not solely due to the ion concentration in the solution, the interaction between solution and tissue also playing a part. The subsequent fall is possibly due to the K^+ penetrating into the leaf.

When KCl and hydroquinone act together (Fig. 5) the two effects are not summated; the hydroquinone, as it were, accentuates the effect of the KCl, perhaps by altering the permeability or the mobility of K^+ in the protoplasm. In unicellular algae the potential is dependent on a single layer of protoplasm; the similarity with our results force us to suppose that the effects we are measuring arise only in the outermost layers of the cells, but that the changes are dependent on the leaf

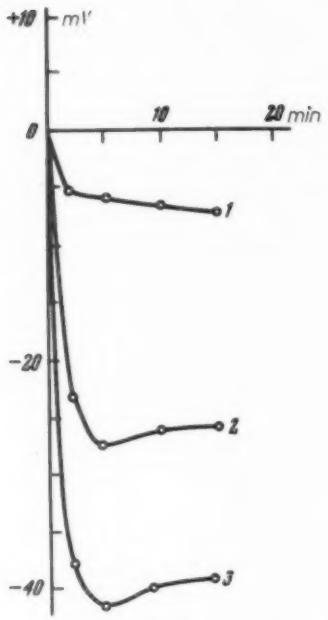

FIG. 4. Potential changes caused by KCl. Each curve is the mean of nine KCl concentrations: (1) 0·01 N; (2) 0·05 N; (3) 0·1 N.

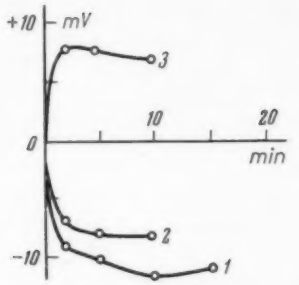

FIG. 5. Potential changes caused by KCl + hydroquinone. Each curve is the mean of five. (1) 0·01 N KCl + 1% hydroquinone; (2) 0·01 N KCl; (3) 1% hydroquinone.

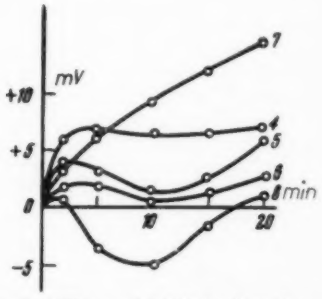

FIG. 6. Effects of 0·2% hydroquinone on leaves of various ages (in triplicate) the leaves are numbered from the bottom of the stem; the seventh had an axial fruit truss.

physiology. Fig. 6 gives results for hydroquinone acting on tomato leaves of various ages; the further down the stem a leaf occurs the greater the positive shift found, the effect in the topmost leaves being sometimes even a slight shift to the negative side. The seventh leaf is an exception, as a fruit truss emerges at this point. These effects coincide with the physiological differences between leaves of different ages, Eh being higher in the top leaves than in the lower ones, while leaves adjacent to fruit trusses usually show lower Eh values.

Hydroquinone and heteroauxin thus produce positive shifts, and H_2O_2 and KCl negative ones. Biologically, heteroauxin acts like hydroquinone (a reductant) and reduces Eh, while KCl in some cases acts like H_2O_2 (an oxidant). The first two compounds retard inflorescence, whereas the latter two may actually accelerate it [1]; we also observed these effects on Eh and inflorescence in our experiments. Here we found that the early potential changes and the effect on Eh and inflorescence were related, but the range of substances used was so small, the potential changes produced by hydroquinone and heteroauxin varied so differently with concentration, and both reductants are unlike the oxidants (having high molecular weights), that it would be premature to say that there is a direct relation between the potential

changes and Eh [4]. It is, however, true that the bioelectric potential changes depend on the nature and concentration of the reagent and on the state of the tissue, so it might be possible to use the effects on the potential produced by various substances to specify the tissue state or the type of action produced by any one substance. The method is of particular interest in relation to studies on the states of protoplasm.

SUMMARY

The leaf potential becomes more positive when heteroauxin and hydroquinone are applied, the reverse occurring with KCl and H_2O_2. The effects are concentration-dependent. The results are determined by the specific actions of the compounds on the protoplasmic state and on ion exchange, and not by damage to the tissues. The potential changes depend on the physiological state of the leaf, the response to hydroquinone being weaker in aged leaves.

Translated by J. E. S. BRADLEY

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THE LAW OF RECIPROCITY AND THE BIOLOGICAL EFFECTS OF FAST ELECTRONS*

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THE primary radiobiological processes are at present inaccessible to observation (neglecting electron paramagnetic resonance methods, which are only just developing), so indirect methods have to be used, wherein the conditions under which the radiation interacts with matter are altered and the changes resultant on this are correlated. One such method is "time factor" analysis, i.e. the dose-rate is altered and the effect on the final result observed (reciprocity law [1]). There are at present two theories of how dose-rate effects arise, which derive from target theory and cumulative action theory respectively.

In target theory [2, 3] multiple hits are considered; to produce a biological effect the target must be hit at several points "simultaneously" (i.e. within the life-time of an active product of radiolysis) by several particles (photons, charged particles, free radicals). The multiple hit probability increases with the radiation intensity, so dose-rate effects should be found. If one hit suffices to produce an effect the dose-rate is unimportant.

In cumulation theory [1, 2, 4, 5] the radiobiological effect depends on two processes, one being the direct formation of radiolysis products and the other the removal of these products, accompanied by recovery in the system. The recovery processes have a larger effect the longer the irradiation time (at a fixed total dose).

Dose-rate effects can thus give data on the detailed primary mechanisms, and can tell us the life-times of the active centres, the rates of recovery processes, etc. As in our earlier work [1, 6] we used wheat-seed shoots, because (a) at 24–48 hr old they are highly radiosensitive, the growth being 50 per cent depressed by 1000 r, (b) the results are very reproducible if standard growth conditions (water, light and temperature) are used, (c) they give an S-shaped dose dependence curve, i.e. they correspond to the multiple-hit model in target theory, and thus to the dose-rate dependence to be expected from that theory. We have previously shown [1] that the effect is reduced by factors of 2–2·5 when total doses of 500–1000 r are given in times of 2 and 430 min; we have also shown that when pulsed irradiation (pulse duration 0·1 sec) is used the effect is the same as for continuous irradiation, if the total dose (500–1000 r) and the total irradiation time (6·2–12·5 min) are the same for both. The dose rate therefore has an effect in prolonged irradiations, but not in pulsed ones, although the instantaneous dose rate in the latter case is 40 times the mean value; but the total dose per pulse was only 2·5 r, giving only about four ion pairs per μ^3,

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so the lack of effect may be due to the low volume density of the ionization. We give here some data on short single shot irradiations performed over a wide range of dose rates, 800 keV electrons being used.

METHODS

A continuously pumped sectional high-voltage tube fed with d.c. from a voltage multiplier circuit was used as electron source. In the first series of experiments the effects of irradiations lasting 20 and 2000 sec (1 : 100) were compared; in the second the times were 0·015 and 1·5 sec (also 1 : 100). The irradiation time at a given dose thus varied over a range of 1 : 130,000. In the first series the time was read from a seconds clock, the dose rate being controlled via the tube current, which was measured by a transmission ionization chamber attached to the outlet window of the tube (see description below); this chamber was calibrated using an electron collector (Faraday cage).

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The irradiations (0·015–1·5 sec) were done with a rotating slit shutter which cut off the electron beam (Fig. 1). The copper disc 5 (with a sector cut out) was coaxial with the duralumin vane 4 and the two were rotated together by a motor, the speed being recorded on a tachometer in the control room. The object was placed under the disc, and was not irradiated while the vane covered the slit; the vane was arrested

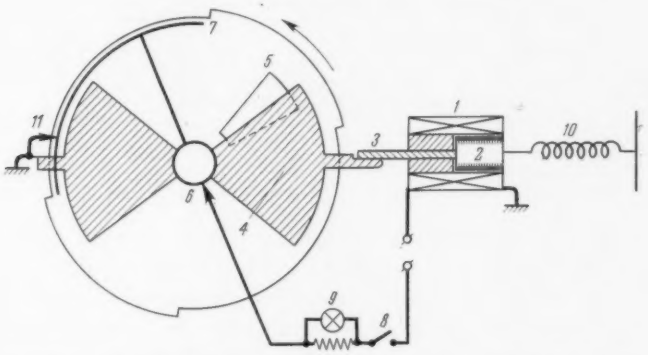

FIG. 1. Rotating slot shutter for single-shot electron-beam irradiations. (1) and (2) winding and core of electromagnet; (3) stop; (4) Al plate; (5) copper disc with slot opening; (6) contact ring; (7) contact arc; (8) and (9) switch and indicator lamp; (10) return spring; (11) contact brush.

when required by an electromagnetic stop, so the sector now travelled through 135° before being again covered by the vane. The automatic stop was released as soon as the vane again covered the sector. The irradiation time at any point on the object was determined by the angular velocity and by the sector angle (15°). The top of the copper disc was covered by a thin sheet of resin-bonded cloth, to reduce X-ray emission from the copper.

The seeds were irradiated 24 hr after soaking, the roots being then not more than 10 mm long; they were kept on moistened paper in a Petri dish during irradiation, and so arranged that the root-tips all pointed upwards without overlapping, so that all were irradiated in the same way. After irradiation the seeds were transferred to sand

and grown in darkess in a thermostat for 6 days; the length of the longest root was then measured, all experiments being done on many specimens.

Emergent Electron Beam Measurements

An ionization chamber, transparent to the fast electrons, was fitted in the beam directly under the exit window; this reference chamber is shown in Fig. 2*A*. The ionization space (5 mm high) was defined by two Al foils, the third (central) foil being the collector. All foils were 12 μ thick; the chamber was flushed with dry air or nitrogen to prevent the foils being distorted by the heat produced at high intensities. This made it easier to get saturation conditions (since the electron capture probability for nitrogen is low [7] and thus easier to work at higher radiation intensities.

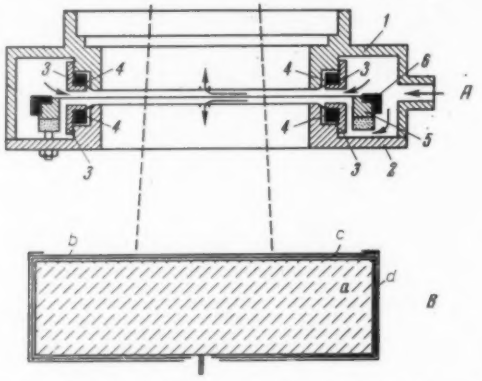

FIG. 2. (*A*) transmission ionization chamber. (1) and (2) body of chamber; (3)–(6) parts of foil tensioning device. The arrows denote the direction of gas flow. (*B*) electron collector. (*a*) graphite, (*b*) Al foil, (*c*) mica, (*d*) earthed outer.

The chamber was calibrated by comparing the ion current with the electron current to the Faraday cage; one design of a cylindrical cage is shown in Fig. 2*B*. A cylindrical graphite block was screened laterally and below by an earthed copper case, and above by a thin foil; the graphite was insulated from the case and foil by 10 μ mica. Fig. 2 shows how the graphite collector was sited under the chamber, the electron beam being shown dashed. (The secondary electron emission from graphite is [8] not more than 5 per cent of the primary electron flux.)

Curve 1 of Fig. 3 shows how the ion current (μA in air) is related to the collector current (in μA); the first is 34 times larger than the second, which agrees well with published values. If the beam cross-section be S, the mean energy flux (in ergs/cm^2 sec) in the electron beam per unit ionization current in air is calculated as follows. The mean current density in the chamber (in e.s.u./cm^3 sec) is $I_v = I_i \times 3 \times 10^9 (0{\cdot}5S)$, I_i being the ionization current in amps. The mean energy flux in ergs/cm^2 sec at 800 kV is $\bar{E} = I_e \times 800 \times 10^3 \times 10^7/S$. Since $I_i/I_e = 34$ we have $\bar{E}/I_v = 39{\cdot}3$ ergs/cm^2 per e.s.u./cm^3. Published data ([7], theoretical, corrected from the experiments of [9]) give 38 instead of 39·3; the 3 per cent discrepancy may arise from a small contribution due to scattering and secondary electron production in the air, foils and mica. Curve 2 of Fig. 3 shows how the ion current varies with the beam current density at 30 cm from the exit window, a 35 mm diameter stop

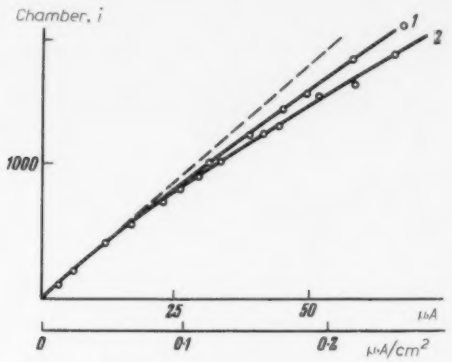

FIG. 3. Relation of chamber and electron collector currents. (1) Total collector current, μA; (2) collector current density, μA/cm^2.

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being placed directly over the collector for this purpose. The voltage applied to the ionization chamber electrodes was 200 V, which resulted in saturation currents at ionization current densities up to 25 $\times$ 10^4 e.s.u./cm^3 sec (as calculated from the current and chamber volume, the chamber being of diameter about 40 mm); this agrees well with published values [10].

The current density was not constant across the beam; it had to be measured and adjusted somewhat, a density meter being used (a miniature graphite collector, similar to that above, with a diaphragm area of about 1 cm^2). The collector was driven along a spiral track of diameter about 200 mm by a selsyn mechanism; measurements made at 30 cm from the tube windows showed that the current density over an object 3 cm in radius did not vary by more than $\pm$ 5 per cent.

Maintenance of Dose Constancy under Different Irradiation Conditions using the Mechanical Shutter

When the 0·015–1·5 sec irradiations were done with the mechanical shutter the dose was calculated as jt, j being the current density and t the irradiation time, known from the shutter speed. An integrating ionization chamber was used to ensure that jt was constant under different conditions; Fig. 4 shows the design. The ionization volume was a flat strip 2 mm thick, the front wall being of tightly stretched 12 μ Al foil, while the rear (collector) wall was of Al backed by polythene; the chamber body was of Al. The beam diameter was adjusted with interchangeable stops; the collector was connected via a 20 m polystyrene insulated cable to a single-fibre electroscope of sensitivity about 15 V/div. The system was charged to 1000 V and the ionization measured in discharge terms; the potential after the irradiation did not fall below 500 V. In all cases where this chamber was used the ion current did not exceed 0·3 μA/cm^3 and was highly saturated (not more than 1·5 per cent of the ions recombine [10].

The integrating chamber was put in place of the object and the readings taken directly before and after the experiment. The divergence between jt and the chamber reading did not exceed $\pm$ 5 per cent.

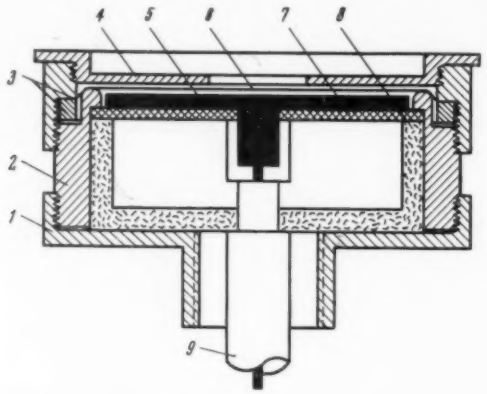

FIG. 4. Ionization chamber for integral measurements. (1) lower cover, (2) body, (3) foil tensioning device, (4) diaphragm, (5) collecting electrode, (6) Al foil, (7) polystyrene insulator disc, (8) perspex, (9) polystyrene insulated cable.

The dose to the object resulting from the X-rays excited during the time the sector was shut was determined with this chamber. Provided certain measures were taken, the X-ray dose was less than 1 per cent of the electron dose.

Calculation of Absorbed Dose

The absorbed dose (in water or water-equivalent material) was estimated from j by calculating the extreme electron range in water, d, from the measured absorption in Al for that energy [11]. The absorbed dose-rate at an electron energy U keV is $P = (Uj \times 10^3 \times 10^7 \times 10^{-6}/10^2 d)$ rads/sec $= (100\ Uj/d)$ rads/sec where j is in μA/cm^2 and d is in cm. We usually used 800 keV electrons, so $d = 0{\cdot}3$ cm, and so $P = 100 \times 800/0{\cdot}3 \approx 2{\cdot}7 \times 10^5$ rads/sec at 1 μA/cm^2.

A more precise estimate would involve using the depth dose curves [12], but here this was unnecessary, since in dose-rate studies only the relative absorbed dose is of interest.

RESULTS

Fig. 5 and Table 1 give the results; the dose curve is S-shaped, the effects (growth retardation as percentage of control) being the same to $\pm$ 5 per cent for all dose rates, the range being 130,000 : 1 (15 msec to 2000 sec). No deviation from the law of reciprocity is observed here; but if an X-irradiation on the same object lasts 4 hr or more, deviations are found [1]. If we assume recovery to follow an exponential law [2] and incorporate the results of [1], we can say that the characteristic recovery time (in which the radiation effect is reduced by a factor (e) is 4–6 hr, which agrees with the findings of others. Kuzin and Strazhevskaya [13] have shown that wheat-seed roots take up less ^{32}P after irradiation, the uptake returning to normal after 4–6 hr. It has also been shown [14] that when 48 hr wheat-shoots are given 500 or 1000 r

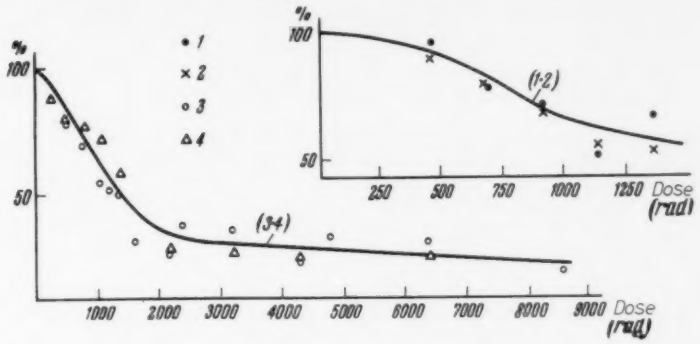

FIG. 5. Wheat rootlet lengths (as percentage control) vs. dose in rads for various modes of irradiation. (1) 0·015 sec, (2) 1·5 sec, (3) 20 sec, (4) 200 sec.

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the mitoses in the root-tips are most depressed at 5–6 hr, a rise following to a maximum at 24 hr.

It has also been shown by electron paramagnetic resonance methods [15] that long-lived radicals are produced in dry barley seeds by irradiation, the radical concentration falling off exponentially. It is quite probable that if the irradiation time

TABLE 1. COMPARISON OF SERIES I (20–2000 SEC) AND SERIES II (0·015–0·5 SEC); LENGTHS OF ROOTS AS PERCENTAGE OF CONTROL

Dose (rads)	500	750	1000	1250
Series I	85	75	65	56
Series II	93	80	67	58
Mean	89	77·5	66	57
Divergence (%)	±4·5	±3·2	±1·5	±1·75

were made comparable with that of the short-lived radicals (OH, HO_2) we might again get a deviation from reciprocity, but there would be considerable technical difficulties in performing such experiments, as millimicro-second techniques would have to be used with high-voltage accelerators.

SUMMARY

(1) The results show that changing the irradiation time by a factor of 130,000 gives no deviation from reciprocity, to ± 5 per cent.

(2) A comparison with previous data [1] for the same object given long X-irradiations (4–8 hr), where deviations from reciprocity were observed, indicates that the plant seedlings recover extremely slowly, so that no deviation from reciprocity is found.

Translated by J. E. S. BRADLEY

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THE ELECTRICAL CONDUCTIVITIES OF LIVER AND SPLEEN HOMOGENATES IN RADIATION INJURY*

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IT has been shown that the livers and spleens of irradiated animals show physico-chemical [1], biochemical [2], pathological anatomical [3] etc. changes, and that the lipids in these organs show some effects not encountered in other organs. Liver homogenates from irradiated mice and rats contain a haemolytic factor [4], which indicates that alterations of molecular structure must occur in the tissues. These changes in molecular structure have been studied [5] by measuring the electrical conductivity of the tissue over a wide frequency range (10^3–10^6 c/s), and it has been found that, with rats and mice, changes of a depolymerization type occur, these appearing as an increase in resistivity and a reduction in dielectric constant. The effects can also be produced by treating fresh tissues with extracts from livers of irradiated animals. Polymerization-type changes have also been shown to occur; these cause an increase in the tangent of the loss angle (tan δ) and the production of filamentary conglomerates in liver homogeneates.

In this paper we consider the conductivities of varies fractions from tissue homogenates corresponding to different stages of radiation sickness. Male rats, 150–200 g, were irradiated with ^{60}Co γ-rays on an EGO-1 unit, at a dose-rate of 32 r/min (total dose 800 r); the animals died 7–8 days after irradiation. Animals were sacrificed 1 day after irradiation, and 1 g lots of liver and spleen homogenized in a glass homogenizer, the homogenate being diluted 1 : 3 with physiological saline. The suspension was centrifuged at 15,000 rev/min for 15 min, and then divided into 3 layers (top, middle and bottom). The specific conductance of the top layer

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was measured at 2×10^4, 10^5 and 10^6 c/s, a bridge circuit, fed from signal generators with a frequency range of 500–2×10^7 c/s, being used. The null detector included a two-stage amplifier, of gain variable from 2×10^2 to 5×10^4, constructed from high-slope triodes and pentodes ($g \approx 10$ mA/V). The accuracy of measurement was to within 5 per cent.

Conductances were measured for whole homogenate (diluted 1 : 3) and for variously diluted centrifugates (1 : 3 by factors of 2 to 1 : 768), directly in the centrifuge tubes immediately after preparation. In all 12 rats (5 experimental, 7 control) were used. The irradiated animals showed no dispersion in homogenate conductance, whereas the controls gave dispersions of 10–13 per cent. The conductance varied somewhat differently with dilution in the liver and spleen homogenates from irradiated animals, a smooth increase in conductance occurring on dilution with saline in the controls, whereas the irradiated animals gave stepwise changes. The conductance increase in the 1 : 6 to 1 : 48 range, then remained constant, and finally increased again at very high dilutions.

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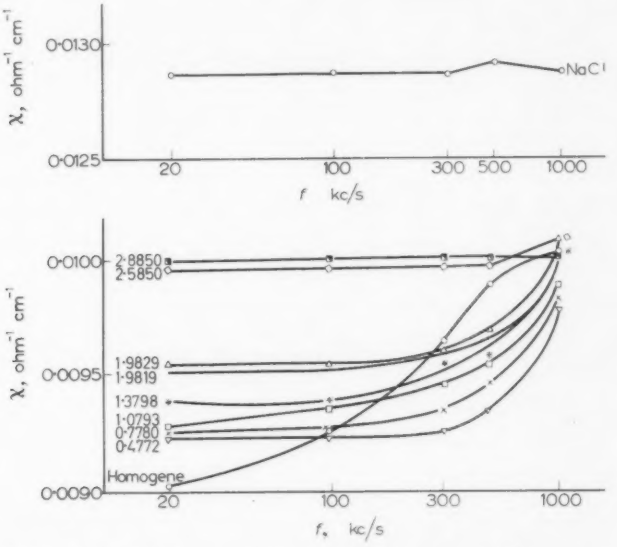

FIG. 1. Relation of $\varkappa$ to f for homogenates of healthy rat liver at different concentrations (C) in physiological saline (NaCl). The figures on the curves are log C.

Figs. 1–4 show the results for liver, those for the spleen being similar; the conductance values for the irradiated animals somewhat exceeded those for the controls, though this was not so at all dilutions and frequencies. Three new phenomena were found with the irradiated animals: the conductance of the top layer is increased over a certain frequency range, the impedance dispersion is reduced or absent, and the impedance varies differently with dilution. The impedances of biological fluids consist of resistive (ionic) and capacitative components [5]; both components show dispersion, that in the capacitative component beginning at a few kc/s, while that in the resistive begins at about 10^5 c/s. Both dispersions are

dependent on particle size, in our interpretation; the dispersion in the resistive component has also been found in erythrocytes and bacteria [6, 7]. As yet there is no agreed interpretation of the components; the subject has been discussed more fully in [5].

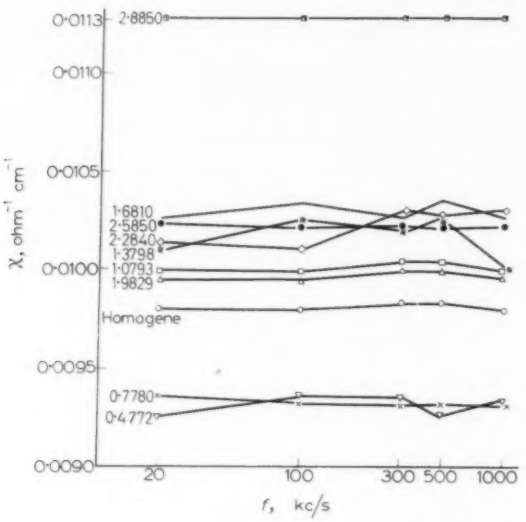

Fig. 2. Relation of x to f for homogenates of livers from irradiated rats.

Our measurements undoubtedly indicate that there is such a dispersion, which is not found over a very wide frequency range with NaCl solutions (physiological saline); in the control animals the dispersion in the resistive component begins at 100 kc/s

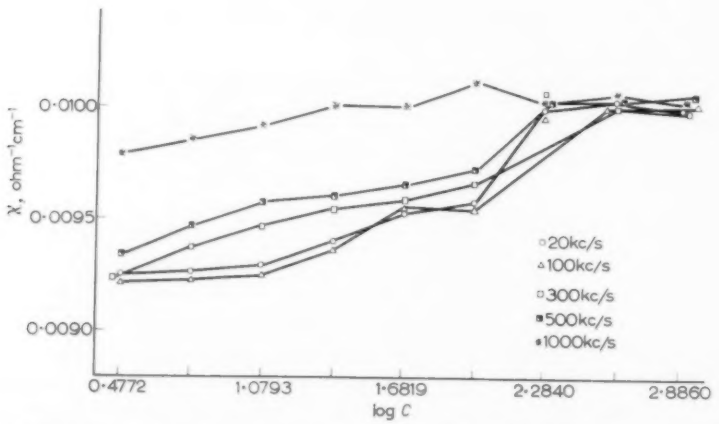

Fig. 3. Relation of x for control rat liver homogenates to C at different concentrations (C) in physiological saline (NaCl).

in the case of the top layer of centrifuged homogenate. The increased conductance found in this layer with the irradiated animals, and the absence of dispersion, would, according to [5], indicate that two related effects are occurring together. The first is that the number of charged particles is increased, the in-phase component I_a being given by $I_a = neu$, where n is the number of ions, e is the ionic charge, and u the ionic mobility. The second is that the particle size or dipole moment is reduced; Figs. 1 and 2 illustrate this very well. Both phenomena can be referred to the same process, i.e. depolymerization (de-aggregation). The anomalous conductance vs. log c relationship for the irradiated tissues is therefore in some way related to changes in molecular structure.

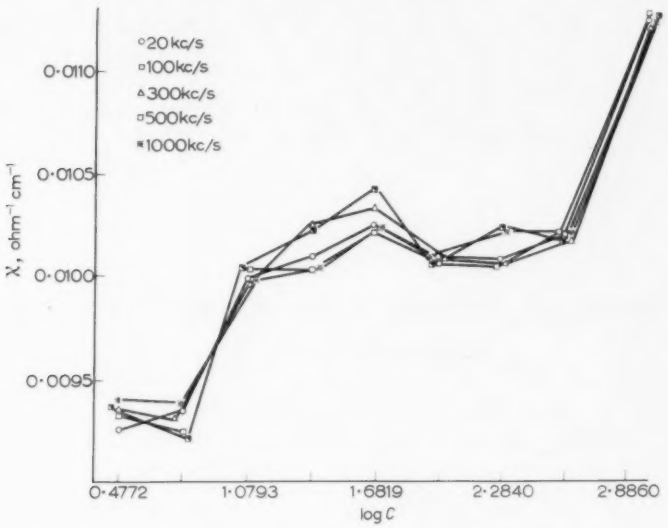

FIG. 4. Relation of $\varkappa$ for homogenates of livers from irradiated rats to C at different concentrations (C) in physiological saline (NaCl).

We only measured the variation of conductance on dilution with an electrolyte; the variation of dielectric constant with dilution with distilled water, using dialyzed homogenates, was not studied. We can therefore only conclude that the number of charged particles is increased, i.e. that depolymerization of some kind occurs. Dielectric constant measurements would enable us to draw more detailed conclusions on the radiation-induced dipole moment changes.

SUMMARY

Depolymerization-type processes occur in the livers and spleens of irradiated rats; these are accompanied by an increase in conductance and the loss of impedance dispersion. The changes appear particularly clearly in the light fraction of the homogenates, and are very marked very soon after lethal doses of ^{60}Co γ-rays. The

lack of dispersion and the anomalous conductance variation with dilution indicate that the dipole moments of some molecular structures must have been altered.

The authors wish to thank B. N. Tarusov, who gave them much valuable advice and assistance.

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THE FUNCTIONAL RADIOSENSITIVITY OF CHLOROPLASTS*

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A KNOWLEDGE of the functional radiosensitivities of the various cell organs is very important to our understanding of the general mechanisms whereby radiation produces its biological effects. The chloroplasts are of interest as they contain numerous enzymes [1] and photosynthesize, thus co-ordinating many enzymes and intermediates which use and transform the light energy. Very little has been published on the effects of ionizing radiations on photosynthesis. In 1921 Henrici [2] studied the effects of radioactive emissions, but his plants were not acted on directly by the rays, so any effect on photosynthesis must be ascribed to air ionization. Stoklasa *et al.* [3] have found that α-rays depress photosynthesis, as do β-rays at first, though a subsequent stimulation occurs after a long time, while γ-rays stimulate photosyhthesis and depress respiration. However, solutions were used in these experiments and no precise dose calculations could be made; the radiation effects could not be distinguished from the chemical effects of the elements acting as radiation sources.

Vlasyuk has given data on radioisotope stimulation of plants; using ^{45}Ca and ^{32}P (0·01–10 μc/16 kg of soil) he found a considerable increase in photosynthesis in the sugar beet [4]. If solutions of ^{45}Ca were applied to the beet leaves at rates of 0·1

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and 0·001 μc/cm^2 the photosynthesis rate in the first few hours was found to be raised by 100–200 per cent [5].

It is very difficult to explain these results, since the radiation doses to the chloroplasts are unknown, as is the localization or length of time of action of the isotopes in the plant. Boichenko and Zakharova [6] state that when radio-carbon is used at a concentration of 0·004% ^{14}C there is no effect on photosynthesis, while if 0·72% ^{14}C is used the rate is depressed three-fold after 6 sec, twelve-fold after 1 min, and completely after 10 min. Nischina *et al.* [7] have found a 45·7 per cent drop in photosynthesis in *Chlorella ellipsoidea* after 3 hr irradiation with neutrons produced by the Be–D reaction in a cyclotron. No effect was found in *Scenedesmus* after a 10 min irradiation, but 7 per cent stimulation was found after 30 min and 37 per cent depression after 2 hr, whereas the respiration was unaffected even by a 3 hr irradiation.

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Morphological studies by Breslavets indicate that *Tradescantia* chloroplasts are stable even to doses of 10–20 kr.* Changes in chlorophyll metabolism on irradiation have been detected; Porter [8] has found that *Chlorella purenoidosa*, grown in water containing 20 μc of ^{3}H per ml, takes up ^{14}C carbonate more slowly than the control. Novotel'nova [9] has studied the effects of X-rays and radon on the pigment system in chloroplasts; at 1500 and 2500 r the protochlorophyll was increased, a slight reduction occurring when the dose was raised to 5000 r or more. It is clear that there are at present no direct data on the effects of X- or γ-rays on photosynthesis.

We used the rate of uptake of $^{14}CO_2$ by intact plant leaves to determine how the functional activity of chloroplasts was affected by irradiation. Preliminary tests showed that the leaves took up negligible amounts of labelled CO_2 (1–2 per cent) in the dark, so the uptake rate in the light could be used to study chloroplast functions.

METHOD

We used leaves from *Tradescantia quianensis* Hort, tobacco (Trapezond 93) and beans (semisweet Gribovskaya); the data of Sparrow and Henkel [10] indicate that *Tradescantia* is the most radiosensitive of these. The leaves were introduced into a special chamber (Fig. 1) via a mercury seal; the chamber contained $^{14}CO_2$, the total CO_2 varying from 0·3–1 per cent. The photosynthesis rate is virtually constant within these concentration limits [11]; the total ^{14}C activity was 10·4 μc in a gas space of 852 cm^3, giving a ^{14}C concentration of 0·004 per cent [6]. The leaves were illuminated by a 300 W filament lamp placed 20 cm away, a water filter being interposed. The leaves were illuminated for 1 hr under the same conditions prior to introducing them into the chamber. The $^{14}CO_2$ uptake rate was determined by a method previously described [12].

After exposure the leaves were fixed in 96% alcohol for 30 min and 14 mm diameter discs cut out from the centres; these were dried between sheets of filter paper pressed between metal plates in a drying oven at 80–100°C for 1 hr. The oven was then switched off and the leaves left in it overnight. The activities were determined using an end-window counter of fixed geometry, two specimens being used per test. Fig. 2 shows the CO_2 fixation rate for *Tradescantia* leaves, and the

* Personal communication from L. P. Breslavets.

nature of the scatter in the experimental data. This scatter compelled us to use 8–24 specimens per point in subsequent work in order to get reliable average figures.

The leaves were irradiated by a RUP X-ray unit at 190 kV, 15 mA (dose rate 334 r/min), the filters being 0·5 mm Cu and 0·75 mm Al; in two cases a ^{60}Co γ-ray unit was used at 600 r/min. Freshly picked *Tradescantia* leaves were placed in moist marl and irradiated. The stems of the tobacco and bean leaves were inserted in tap water, the ends of the stems being cut off immediately after the irradiation. The leaves were then placed in tap water and left for a set time in darkness. An hour before

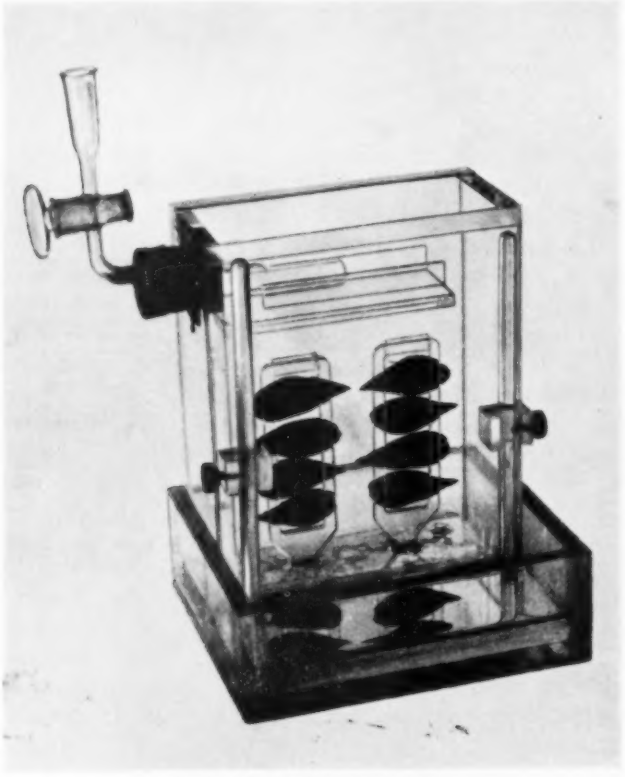

FIG. 1. Photosynthesis chamber.

the uptake determination the leaves were illuminated as described above. As the CO_2 uptake rate was always represented by a straight line we used the uptake in 15 min as index. In all cases control leaves were used under precisely the same conditions, apart from irradiation. In the *Tradescantia* experiments we used young leaves (second or third set reckoned from the tip) and older ones (seventh to eighth set).

RESULTS

The data on CO_2 uptake for leaves of different ages from the various plants are summarized (after statistical treatment) in Table 1 (some 800 determinations were used). It is clear that even the radiosensitive *Tradescantia* shows no statistically

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TABLE 1. RELATIVE ACTIVITIES OF LEAVES FROM *Tradescantia* (YOUNG AND OLD), TOBACCO AND PEAR AFTER 15 MIN PHOTOSYNSYNTHESIS IN A $^{14}CO_2$ ATMOSPHERE

Plant	Dose (kr) and radiation type		Time after irradiation (hr)					
			3		24		48	
			Experimental	Control	Experimental	Control	Experimental	Control
Tradescantia young leaves	20, RUP	Count rate (min^{-1}) Error (%) Count rate as % of control	— — —	— — —	3687±257 ±7 99·6	3700±219 ±6 100	3400±66 ±2 91·9	3700±201 ±5 100
	50, RUP	Count rate (min^{-1}) Error (%) Count rate as % of control	3047±228 +7 87	3500±303 +8 100	1190±420 +35 32	3722±220 +6 100	588±195 +33 18	3261±202 +6 100
Tradescantia old leaves	50, RUP	Count rate (min^{-1}) Error (%) Count rate as % of control	2433±488 ±20 100·07	2414±198 ±8 100	1386±245 ±17 54	2539±135 ±5 100	1275±402 ±31 46	2758±296 ±10 100
Tobacco, 2·5 day leaves	50, RUP	Count rate (min^{-1}) Error (%) Count rate as % of control	—	—	5455±525 ±10 85	6393±294 ±5 100	3908±263 ±7 87	4477±290 ±6 100
	50, γ-rays	Count rate (min^{-1}) Error (%) Count rate as % of control	— — —	— — —	2116±186 ±8 82	2695±162 ±6 100	2386±321 ±13 98	2451±243 ±10 100
Bean, 9 day leaves	50, γ-rays	Count rate (min^{-1}) Error (%) Count rate as % of control	—	—	4726±856 ±18 82	5724±914 ±16 100	2427±316 ±13 92	2641±444 ±16 100

significant deviations from the control at 24 or 48 hr after irradiation, even at 20 kr. The differing radiosensitivities are apparent at 50 kr, but the tobacco and bean are hardly affected even at this level. The results for the young and old *Tradescantia* leaves are particularly interesting, since no effect appears at 3 hr with the old leaves, while the young show a 13 per cent drop in activity, this fall becoming 68 per cent at 24 hr, but being only 46 per cent in the old; the values for 48 hr are 82 and 54 per cent respectively. It is noteworthy that the scatter in the control values is 5–10 per cent, but in the irradiated leaves it rises to 35 per cent; this change has not been found in relation to other functions of the irradiated plants.

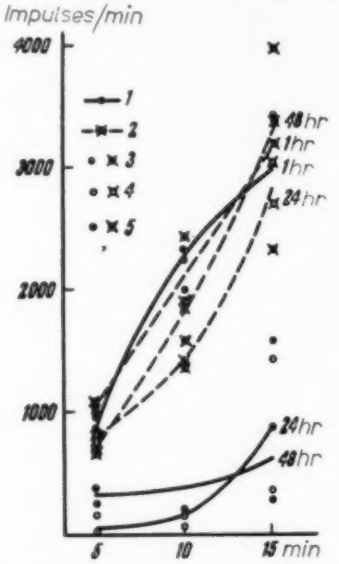

FIG. 2. $^{14}CO_2$ uptake curves. (1) Experimental, (2) control, (3) 1 hr after irradiation, (4) 24 hr after irradiation, (5) 48 hr after irradiation.

The data show that the chloroplasts are more radiosensitive (as regards their functional activity of photosynthesis) than are the other cellular functions, such as nuclear division, or oxidative phosphorylation in the mitochondria. The effect, as with other functions, appears gradually, appearing at 3 hr and increasing at 24 and 48 hr. This time course is typical of depolymerization processes in macromolecules, particularly the nucleic acids; high nuclear radiosensitivity is coupled to high DNA content (20–38 per cent of the dry weight [13]. The mitochondria also contain relatively large amounts (6–10 per cent) of RNA, while the chloroplasts contain only 0·5–3·5 per cent nucleic acid [14], which may be related to their high radioresistance. It was of interest to check this by determining the nucleic acid contents of chloroplasts from young and old *Tradescantia* leaves, since these were found to have different functional radiosensitivities.

A difficulty here is to obtain a chloroplast suspension free from cell debris, nuclei and mitochondria; we used Hagendorff's method [15], somewhat modified. The leaves (5 g) were homogenized in 25 ml of phosphate buffer, of pH 6·89, which was

0·4 M in sucrose, the homogenate being centrifuged at 1000 rev/min for 10 min. The deposit was again treated in the same fashion, the supernatants being combined and centrifuged at 2000 rev/min for 24 min. The chloroplasts were thus thrown down; they were suspended in 10 ml of phosphate buffer, to which was added an equal volume of a 5 : 3 mixture of gylcerol and a sucrose solution containing 1·3 g of sucrose per 3 ml. The suspension was centrifuged for 20 min at 8000 rev/min, the deposit being resuspended in the same solution and centrifuged as before. The deposit was washed several times with phosphate buffer, until the nuclear debris was removed. The chloroplasts were then carefully washed twice with distilled water.

The chloroplasts were examined under the microscope (kindly undertaken by Dr. Breslavets) and were found to be entirely free from nuclei, etc. After drying *in vacuo* the nucleic acid was determined by the method of Ogur and Rosen [16]; the results are given in Table 2.

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TABLE 2. NUCLEIC ACID CONTENTS OF YOUNG AND OLD *Tradescantia* LEAVES

Source	Experiment No.	Nucleic acid contents (%)	Content excess of young over old (%)
Young leaves (second or third from tip)	I	2·56	127
	II	2·28	116
Old leaves (seventh or eighth from tip)	I	2·02	100
	II	1·96	100

There is clearly a good correlation between nucleic acid content and radiosensitivity in these chloroplasts, the young leaves being more radiosensitive and containing more nucleic acid.

SUMMARY

(1) The effects of ionizing radiation (X- and γ-rays) on the photosynthetic activites of chloroplasts in intact leaves from *Tradescantia* tobacco and bean have been studied. The photosynthetic activity was determined from the rate of uptake of $^{14}CO_2$.

(2) The chloroplasts have been shown to be functionally highly radiosensitive: 20 kr produced no effect on any of the plants, but 50 kr affected the *Tradescantia*; these leaves are more radiosensitive and show reduced activity. The activity reduction became greater with the passage of time and was very marked at 24–48 hr after irradiation.

(3) The chloroplasts in young leaves were more radiosensitive than those in old ones, young *Tradescantia* leaves showing activity reductions after 50 kr of 13 per cent after 3 hr, 68 per cent after 24 hr and 82 per cent after 48 hr.

(4) The low radiosensitivity of the photosynthetic activity in chloroplast is considered to be due to the low nucleic acid content.

(5) Nucleic acid determination on pure isolated chloroplasts show that more nucleic acid is present in the more radiosensitive young leaves.

Translated by J. E. S. BRADLEY

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RADIATION PROTECTION AGENTS AND MORTALITY PEAKS*

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WE have shown earlier [1] that the mortality rate of irradiated animals does not follow a normal law, there being 5 maxima (mortality peaks); the causes of the peaks were assumed to be different ultimate causes of death. The present paper gives data in support of this assumption; one of the methods used was to determine the effects on the peaks of injecting the animals with various protective agents. If some substance selectively influences the mortality distribution it is clear evidence that the various peaks have different ultimate causes; but a simple calculation shows that at the $LD_{50/30}$, at which all 5 peaks are about equally prominent, we would have to use

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hundreds of animals in order to establish a statistically reliable redistribution. We can avoid this by using higher doses, so all animals die in the early stages (covering only the first 1–2 peaks). The results are also less variable at high doses [4], and some authors have therefore proposed that protective agents should be tested at high doses [3]; however, this is rarely done, probably because most of the substances which reduce the mortality at lower doses are ineffective at high doses [4]. We therefore decided to limit our comparative studies on a large range of materials to the first two peaks, and these experiments form the main topic of this paper. The deductions are also checked with a few agents in experiments covering all five peaks.

We have already shown that the first mortality peak ends at about day 5 [1]; Table 1 gives some data on the relation of peak 1 mortality to dose, whence it is clear that the peak 1 mortality at low doses is dependent on the strain of mice used, but that at 900–1000 r virtually all deaths begin to fall in peak 1, and at 1000 r strain, sex and age have little effect on the mortality, although they have a major influence on the $LD_{50/30}$ [5]. Our main experiments were done on 1260 strain H mice, using 1000 r ^{60}Co γ-ray doses, at dose-rates of 38–45 r/min, 300 control mice being used. Fig. 1 shows the peaks for the controls, only the first three appearing at 1000 r; the limits to the first and second peaks can be set at $5\frac{1}{2}$ and $7\frac{1}{2}$ days. Of the animals irradiated 86·3 per cent died in peak 1, 12·1 per cent in peak 2 and 1·6 per cent in peak 3, all being dead by the tenth day. Several experimental groups and one control

TABLE 1. PEAK 1 MORTALITIES OF VARIOUS MOUSE STRAINS AT VARIOUS DOSES

Dose, r	Strain								
	X			M			H		
	No. of mice	Dying in first peak		No. of mice	Dying in first peak		No. of mice	Dying in first peak	
		(No.)	(%)		(No.)	(%)		(No.)	(%)
400	50	2	4·0	—	—	—	36	0	0
500	397	47	11·8	32	0	0	66	0	0
600	70	11	15·7	339	47	13·9	134	11	8·2
700	60	16	26·7	190	48	25·2	376	54	14·4
800	15	8	53·3	23	7	30·4	76	22	29·0
900	13	12	92·3	33	23	69·7	34	17	50·0
1000	12	12	100·0	16	13	81·2	180	161	89·5

group were irradiated together, so each set had its own control. Fig. 2 compares the mortalities for 80 control groups (12 in each) with a Poisson distribution; there is no real difference between the two, since $\chi^2 = 6{\cdot}01$, $n = 3$, $p = 0{\cdot}11$. The results were therefore considered using a combined control. Because many agents were to be tested it was desirable to keep the number of distinct groups to a minimum, and 12 mice were used with each agent, since a 50 per cent fall in peak 1 could be established with certainty on this number; as the numbers were small, care was taken to use Fisher's precision methods [6]. Special binomial expansion tables were compiled from the control results for use in these experiments.

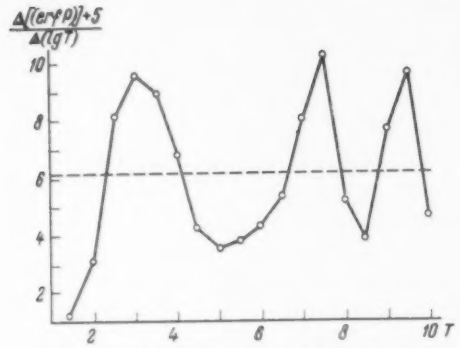

FIG. 1. Mortality peaks for strain H mice at 1000 r γ-ray doses. The increments in the mortality probits are plotted against log T (T = time after irradiation).

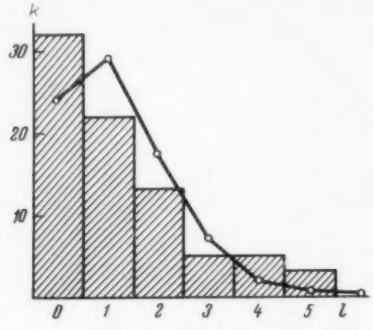

FIG. 2. Distribution of the number (k) of control groups in the various experiments vs. number of surviving animals (at end of peak (l)). The rectangles are the actual distributions, the curve being the Poisson distribution for $m = 1{\cdot}2$.

One aim in the experiments was to test substances of widely differing types, so the agents were classified phenomenologically in terms of the optimum time for use [7]. In this classification all agents are divided into four types: prophylactic, i.e. effective if used several days before irradiation (e.g. foreign proteins [8], oestrogens [9], low-dose irradiation [10]); protective, if effective when used immediately (such as sulphydryl compounds [11], ethanol [12] etc.); therapeutic, i.e. effective if used after irradiation (antibiotics [13], spleen and bone-marrow preparations [14, 15]); and background, i.e. ones effective if applied for a long period (adrenal cortical hormones [16], certain vitamins [17]). In our experiments we tested 80 such agents: 10 prophylactic (used 10 days before irradiation), 50 protective (used directly before irradiation) and 20 therapeutic (used immediately after irradiation). So far as possible we tested almost all the prophylactic and therapeutic agents given in the literature or known from our own experiments; as regards protective agents, we chose a few representative ones, since these are very numerous. We did not use background agents, since no effect from these is to be expected at such early stages. All materials were injected once only, using different methods. As the conditions were very severe 48 of the 80 agents were without effect on survival; of these 48 most are effective at lower radiation doses, so the negative results are of interest as showing either that these agents act by reducing the mortality in the later peaks, or that they have little effect on the first peak (which effect therefore cannot be demonstrated on few animals). The following prophylactic agents were ineffective: histamine, horse serum, methyltestosterone, milk, progesterone and urethane. The ineffective protective agents were: adrenalin, aconitic acid, ascorbic acid, acetone, hexanal, histamine, dimexdrol, gelatin, insulin, KCl, campolone, cobalmin, morphine, niacin, nitrite, *p*-phenylenediamine, pyridoxin, riboflavin, tannin, thiamin, urethane, folliculin, folic acid, fumarate, quinosol, KCN, ethanol, ethylenediamine and ephedrine. The ineffective therapeutic agents were: atropine, deoxycorticosterone, yeast extract, carbocholine, lecithin, methyltestosterone, miol, RNA, spleen homogenate, streptomycin, $HgCl_2$, cholesterol and cysteine.

TABLE 2. EFFECTS OF PROTECTIVE AGENTS ON THE MORTALITY FOR MICE GIVEN 1000 r γ-RAY DOSES

Agent	Dose		Total No. of mice	Surviving peak 1	Surviving peak 2	Surviving peak 3	P_1	P_2	P_3	P_{1+2}	P_{1+2+3}
A. Use 10 days before irradiation											
Diethyl-sulboestrol	0·1 mg	SC, IP	12	11	11	3	**0·92**	**1·00**	0·27	**0·92**	**0·25**
γ-irradiation	100 r	—	12	6	4	0	**0·50**	**0·67**	0	**0·33**	0
Folliculin	1000 units	SC, IP	10	8	5	1	**0·80**	**0·63**	0·20	**0·50**	**0·10**
Embikhin	1–5 mg	IV	10	8	5	0	**0·80**	**0·63**	0	**0·50**	0
B. Use directly before irradiation											
Alanine	5 mg	IP	12	2	2	2	0·17	**1·00**	1.00	**0·17**	**0.17**
Anilide of thio-glycollic acid	3 mg	,,	12	3	2	0	0·25	**0·67**	0	**0·17**	0
Acenaphthene	1 mg	,,	8	4	0	0	**0·50**	0	0	0	0
Berberine	0·1 mg	,,	12	5	3	1	**0·42**	**0·60**	0·33	**0·25**	**0·08**
$Na_2S_2O_3$ $NaHSO_3$	10 mg	,,	12	3	3	0	0·25	**1·00**	0	**0·25**	0
$Na_2S_2O_3$ $NaHSO_3$	20 mg	,,	12	3	2	0	0·25	**0·67**	0	**0·17**	0
Glycine	5 mg	,,	12	3	2	0	0·25	**0·67**	0	**0·17**	0
Glucose	50 mg	IV	8	4	0	0	**0·50**	0	0	0	0
Caffeine	2 mg	,,	12	3	2	0	**0·25**	**0·67**	0	**0·17**	0
Lysine	5 mg	IP	12	7	0	0	**0·58**	0	0	0	0
Horse serum	0·5 ml	,,	11	5	1	0	**0·45**	0·20	0	0·09	0
Na pyruvate	5 mg	,,	12	10	6	2	**0·83**	**0.60**	0·33	**0·50**	**0·17**
Serine	5 mg	,,	12	2	2	0	0·17	**1·00**	0	**0·17**	0
Strychnine	0·05 mg	PO	5	4	2	0	**0·80**	0·50	0	**0·40**	0
Thiourea	20 mg	IP	11	4	3	0	0·36	**0·75**	0	**0·27**	0
Na acetate	5 mg	,,	12	5	1	0	**0·42**	0·20	0	0·08	0
Cysteine	15 mg	,,	11	5	2	1	**0·45**	0·40	0·50	0·18	**0·09**
Na citrate	5 mg	,,	12	3	3	1	0·25	**1·00**	0·33	**0·25**	**0·08**
Na ethylene-diamine tetra-acetate	2 mg	,,	12	5	5	1	**0·42**	**1·00**	0·20	**0·42**	**0·08**
Na malate	5 mg	,,	12	5	5	1	**0·42**	**1·00**	0·20	0.42	**0·08**
Succinic acid	5 mg	,,	12	5	4	0	**0·42**	**0·80**	0	**0·33**	0
C. Use immediately after irradiation											
Anilide of thio-glycollic acid	3 mg	IP	12	4	2	0	0·33	0·50	0	**0·17**	0
Acenaphthene	1 mg	,,	12	2	2	0	0·17	**1·00**	0	**0·17**	0
Berberine	0·1 mg	,,	12	5	4	1	**0·42**	0·80	0·25	**0·33**	**0·08**
Novocaine	1 mg	,,	11	6	3	2	**0·55**	**0·50**	0·67	**0·27**	**0·18**
Liver extract	0·1 mg	,,	11	3	2	1	0·27	**0·67**	0·50	**0·18**	**0·09**
Na pyruvate	5 mg	,,	12	5	2	0	**0·42**	0·40	0	**0·17**	0
Pituibin	0·6 unit	IV	12	4	2	0	0·33	0·50	0	0·17	0
Control	—	—	300	41	5	0	0·14	0·12	0	0·02	0

Notes. The doses are per 20 g mouse. Methods: IP = intraperitoneal, IV = intravenous, SC = subcutaneous, PO = per os.

In all cases 12 animals were used, the fourth column giving the number surviving after using the agent and irradiating, since the doses were sometimes toxic, or accidents occurred (embolism, shock, death during irradiation).

P_i is the probability of death in peak i.

The bold figures denote values showing a statistically reliable deviation from the control, to 95 per cent.

Table 2 gives the results where an effect was found; of the 32 effective agents eight selectively reduce peak 1, eleven peak 2 and eleven both peaks. Two had no real effect on either peak in isolation, but reduced the total mortality for the two peaks. The results will be considered from the viewpoint presented above.

If the peaks did not represent distinct causes of death the various agents should only extend the survival time to a greater or lesser extent, so the mode should be displaced to the right and the whole distribution become flatter than for the control.

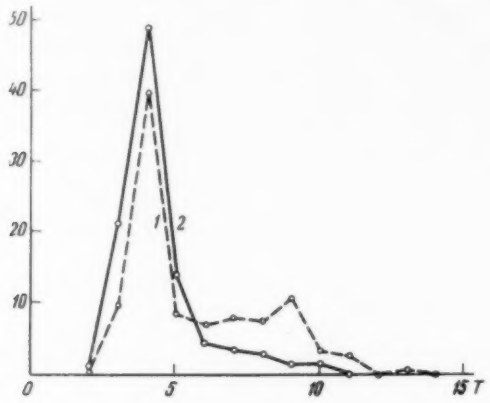

FIG. 3. Mortality distributions for mice receiving an effective protection agent (1) and for the corresponding control mice (2) (T = time after irradiation).

Fig. 3 shows the combined data for all the effective protective agents and the distribution for the corresponding controls. The mode clearly remains at the same point, although the mean life-span and the dispersion are, formally speaking, increased; but for peak 1 the mean life-span and dispersion are unchanged. A point of particular interest is that the test animals showed more deaths in days 7–9, this being the period of the second peak [1]. We can interpret this as animals which have survived the first critical period having a greater probability of dying in the second.

We have already seen (Table 2) that about equal numbers of agents affect the first and second peaks selectively, but as the numbers are small this selectivity may only be apparent, since it may arise from random fluctuations. In Table 3 we

TABLE 3. DISTRIBUTION OF AGENTS IN TERMS OF SELECTIVITY FOR VARIOUS P VALUES

	P			
Peak	0·1	0·05	0·01	0·001
1	7 (22%)	8 (26%)	3 (20%)	4 (57%)
2	10 (31%)	11 (37%)	7 (47%)	2 (29%)
1 and 2	15 (47%)	11 (37%)	5 (33%)	1 (14%)
Totals	32	30	15	7

compare the effects of the various agents; it is clear that the selectivity actually increases with the probability, since the fraction of substances reducing both peaks falls from 47 to 14 per cent. The selectivity does not tend to centre on one peak. Although the various substances affect the various peaks differently, the peaks are not entirely independent: 19 agents reduce peak 1 alone, or both, 22 reduce peak 2 (out of 80). If the effects on the peaks were strictly independent the number of agents affecting both peaks should be given by the products of the probabilities, i.e. should be 5, whereas actually it is 11, i.e. more than twice the mathematical expectation.

TABLE 4. DISTRIBUTIONS OF AGENTS AS REGARDS SELECTIVITY AND TIME OF USE

Time of use	Agent		
	Peaks reduced		
	One	Two	One and two
Ten days before irradiation	—	—	Diethyl stilboestrol Low γ-ray doses Folliculin Enbihin
Directly before irradiation	Acenaphthene Glucose Lysine Horse serum Strychnine Acetate Cysteine	Alanine Anilide of thioglycollic acid $NaHSO_2$ $Na_2S_2O_3$ Glycine Caffeine Serine Thiourea Citrate	Berberine Pyruvate EDTA Malic acid Succinic acid
After irradiation	Pyruvate	Acenaphthene Liver extract	Berberine Novocaine

The calculation was done for $P = 0{\cdot}05$, comparable results being obtained for other values of P; this is because, while some agents act selectively on one peak, others affect several. This is only to be expected, since such agents must act by "reducing the effective dose" [7]. It would appear that all the effective prophylactics appear to act similarly, at least as regards the first two peaks; this would agree with the idea that they act by raising the general resistance [8, 9].

It is of some interest to consider some of the agents in detail; in Table 4 we have grouped together all effective agents under the headings of time of use and peak affected. The distribution is clearly not random, since of the 9 protective agents which selectively reduce the second peak, 7 are amino acids or sulphur compounds, while of the 12 which reduce the first peak (selectively or not) 6 fall in the Krebs' cycle. This confirms our idea that the peaks reflect differing ultimate causes of death; the analysis can be made the basis for classifying the agents. A classification of this type will resemble Table 4, the broad groups, which relate to optimum time of application, being subdivided in terms of the mortality peaks affected. Table 4 itself does not

constitute such a classification, because few animals were used per test, and a more detailed study might result in certain changes; but the experimental data are sufficiently extensive to give statistically valid general conclusions.

It would be of great interest to examine these numerous agents in relation to all five mortality peaks, but here doses of $LD_{50/30}$ order would have to be used, and very many animals would be needed. Although much has been published on protective agents, and many animals have been used in some tests, the material is often presented in such a way that it cannot be analysed in terms of mortality peaks. We give below some of our own data on the effects of various factors on the mortality peaks at lower doses. These experiments (Table 5) were done in relation to studies on the agents, and have in part been described in more detail [8, 10, 12]. Each group given in Table 5 consists of several slightly different modifications, the variations being in

TABLE 5. EFFECTS OF VARIOUS FACTORS ON THE MORTALITY OF MICE GIVEN 500–700 R γ-RAY DOSES

Factor and time of use	No. of mice	Dead by day 20		Differences		P
		No.	%			
Cysteine before irradiation	118	50	42·4	28·8	20·0	1×10^{-6}
Control	118	84	71·2			
Folliculin or diethylstilboestrol	106	43	40·5	38·7	32·9	1×10^{-8}
Control	106	84	79·2			
Yeast extract after irradiation	219	128	58·5	15·8	15·5	8×10^{-5}
Control	346	257	74·3			
Atropine before or after irradiation	120	56	46·7	13·3	4·3	0·028
Control	120	72	60·0			
Thiourea before irradiation	117	75	64·1	16·2	7·7	0·0055
Control	117	94	80·3			
Ethanol before irradiation	78	32	41·0	24·4	9·3	0·0023
Control	78	51	65·4			
Small-dose irradiations 10 days previously	91	45	49·5	29·6	17·4	3×10^{-5}
Control	91	72	79·1			
KCN before irradiation	96	57	59·4	15·6	5·3	0·021
Control	96	72	75·0			
Dose-rate 10 r/min; M strain mice, 500 r	257	154	60·0	12·2	5·4	0·020
Dose-rate 50 r/min,	122	88	72·2			
H strain males, 700 r	150	104	69·4	13·3	7·3	0·0070
H strain females, 700 r	150	124	82·7			

factors such as dose of agent, radiation dose, sex and strain; the figures for the mean reduction in mortality are therefore sometimes lower than the best (sometimes very much so). The data are quite adequate for our purpose, since adequate controls were always used. The results were drawn up as previously [1].

The P_t values (probability of death within a peak) were calculated for the five peaks (0–5, 6–9, 10–12, 13–15 and 16–20 days). Fig. 4 shows the differences between

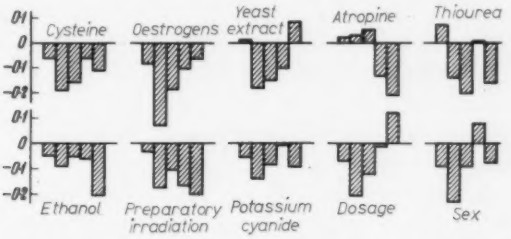

FIG. 4. Effects of various agents on the mortality peaks at 500–700 r. The differences of the P_t values for the experimental and control animals are shown for five peaks.

the P_t values for the experimental and control groups; when the factor is not a protection agent (e.g. dose rate and sex) the control is taken as being the group

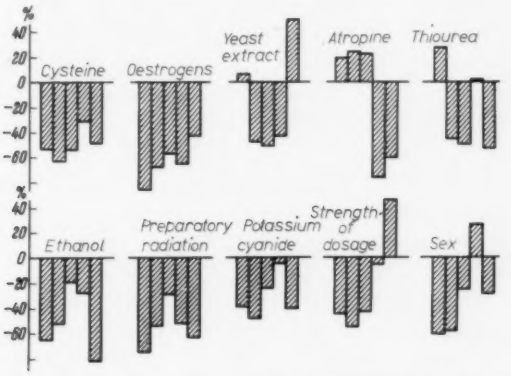

FIG. 5. Effects of various agents on the mortality peaks at 500–700 r.

of higher mortality. This method is straightforward, but the results are very much affected by the absolute mortality in the control. The effect is eliminated if ratios are used instead of differences, as in Fig. 5. If we compare both figures we see that the various factors affect different peaks; thus cysteine and the oestrogens reduce all peaks more or less equally, prior irradiation reduces the early deaths, atropine reduces the late deaths, and yeast extract the median ones. Ethanol and KCN appear to give two optima. In no case is absolutely strict selectivity found. Since the peaks overlap to a considerable extent [1, 5] a reduction in one peak must affect the neighbouring ones; a reduction in one pathological process should, after all, affect the probability of death from other causes. The general pattern of these results is similar to that for the higher doses, and all our data given here show that protective agents have different

"action spectra" against the mortality peaks. This confirms our view that the various peaks have different ultimate causes.

These peaks can be used in a phenomenological classification of protective agents, and the data can also assist us in understanding how particular agents act. Table 4 shows that substances of similar biological and pharmacological properties frequently give similar "action spectra". Substances showing the same "spectrum" can be compared in properties, and the important properties thereby established, though this topic falls outside the confines of this paper, which deals mainly with the first peak, on which we have the most data. The substances most effective in reducing the first peak are notably those which are related to the Krebs cycle, so disturbance to this cycle, or to some closely related biochemical processes, may be responsible for the early deaths. The most likely interpretation of how prophylactics act is that they increase the general resistance.

In conclusion we must emphasize that our conception of the mortality peaks must not be over-simplified, since no one peak is reduced in strict isolation, and some peaks may be composite, since several pathological processes may be involved. Mortality peak analysis is not self-sufficient, but must be supplemented by other methods in order to give a precise objective picture.

SUMMARY

(1) Experiments on the effects of various factors on the mortality peaks appearing in the post-irradiation death distribution are described; mice irradiated with ^{60}Co γ-rays were used.

(2) The protective action of 80 agents was tested on the first and second peak at 1000 r in the main experiment. Of these, 8 selectively reduced the first peak, 11 the second, and 11 both.

(3) A similar picture is obtained at lower doses, at which five peaks appear; some agents reduce all five, others are somewhat selective.

(4) It is concluded that the peaks reflect different ultimate causes of death, and that the various protection agents act in different ways.

In conclusion I express my thanks to N. V. Timofeev-Resovskii, A. A. Lyapunov and N. A. Poryadkova for their interest in the work, and for some valuable advice.

Translated by J. E. S. BRADLEY

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METHODS AND APPARATUS

PRESENT-DAY APPARATUS FOR CYTOSPECTROPHOTOMETRY*

L. S. AGROSKIN

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CASPERSSON (Sweden) performed the first experiments on the absorption spectrophotometry of biological objects in 1936; he demonstrated that the inherent absorptions of cells and tissues in the ultra-violet could be measured. His apparatus consisted of a quartz-optics microscope, monochromator and photometer, and was far from ideal, but the results were of great interest, and stimulated developments in the theory and techniques of cytospectrophotometry. Brodskii has recently reviewed the cytospectrophotometric work of the last 20 years [1]; he mainly dealt with theory and methods. Some data on apparatus, as well as from research studies, are given in [2–5]; here we describe cytospectrophotometric apparatus described in the literature in the past 5–6 years. Only photoelectric devices are considered, since photographic methods are well known and have not materially altered in recent years.

Most arrangements for ultra-violet absorption cytophotometry consist of light sources, monochromator, microscope and radiation detector. A cytospectrophotometer differs from a normal spectrophotometer in having a microscope set up to give an image of the specimen; certain components demanded by the special properties of biological objects are also included. These properties are as follows.

(2) Cytochemical spectrophotometry uses the Beer–Lambert law, which assumes the absorbing centres uniformly distributed over the whole light beam, whereas a real cell is complex, consisting of particles of various shapes and sizes. Errors arise if the average is taken over large areas; these increase as the particles grow smaller, or their concentration higher. The photometered area is usually delimited by a stop in the image plane, in front of the photocell; the equivalent diameter of the stop in the object plane is about 1 μ. The optical density (O.D.) and distribution in such an area are commonly uniform [1]. This can be termed pointwise photometry.

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(2) One-point readings seldom suffice in cytochemistry, so numerous readings are usually taken, evenly spaced over the specimen, or lying on a straight line. This is successive cytophotometry [6–10], and special devices for the purpose have been made, which operate by scanning, coupled to continuous absorption recording.

(3) Live objects may be damaged by ultra-violet light; cells are rapidly killed by strong ultra-violet. Photochemical changes can occur and change the original absorption in fixed cells. This has to be considered in designing cytospectrophotometers.

Light Sources and Monochromators

Two types of work are done on cytospectrophotometers.

(1) The absorption spectrum at a point is taken.

(2) The O.D. (distribution of absorbing material) along a section is recorded.

In the first case a continuous-spectrum source is needed; in the second a line spectrum source can be used, since fixed wavelengths are used. Filament lamps are most commonly used in the visible; they are suitable for stained preparations [9, 11–13]. The ultra-violet region is, however, of more interest in cytophotometry, particularly below 360 mμ. Superhigh pressure Hg lamps are usually used; although a line spectrum is emitted, the source is suitable for much work, because three bright lines (280, 265 and 248 mμ) fall in the most important range. The Phillips capillary lamp of this type is unequalled down to 360 mμ, since it is very bright and has an intense background continuum.

For work below 260 mμ Caspersson [10] suggests a Kohler high-voltage condensed-spark; this burns between rotating disc electrodes and emits a line spectrum dependent on the electrode material. In the ultra-violet region 20 different bright lines can be had by using Cd, Ni, Mg, Al and Zn; Cd electrodes are particularly suitable, since the 257 and 275 mμ lines fall near the maxima of the nucleic acids and of certain amino acids. The situation is less happy with sources of continuous ultra-violet light, there being none of adequate intensity. Xe- or Kr-filled lamps are usually used down to 250 mμ [9, 14, 15]; for shorter wavelengths some workers propose hydrogen discharge tubes [2, 9, 10, 16], but these are of such low intensity that only large areas of the specimen can be photographed.

The use of intense sources might seem at first sight to conflict with the need to reduce radiation dose to the object, but the need for bright sources is evident if we remember that the area used is often less than 1 μ^2; the risk of damage is reduced by decreasing the exposure time [17]. In scanning arrangements the source output must be constant, otherwise the density curve will be distorted. The best way of eliminating the effects of source instability is to use a double-beam system, as in [4, 15, 18]. In single-beam systems the light sources have to be stabilized, e.g. Deeley [19] has kept the light flux constant electronically, using a circuit which shunts the mercury lamps when the voltage rises. The SVD-120 (superhigh pressure) lamps burn quite stably when fed through resonant-core stabilizers; this reduces the brightness fluctuations to 1 per cent [14].

Normal methods (filters, prism or grating monochromators) are used to isolate the required spectral regions. Glass filters are used with visible and stained preparations, but large errors can result from using wide-band filters with continuous sources,

particularly at high O.D. [20]. Narrow pass-band interference filters or monochromators are better used in such cases. It is only necessary to isolate lines lying close together in the visible when, for instance, changes in the stain spectrum are being studied. In the ultra-violet region high spectral purity, coupled to measurements at adjacent wavelengths, is almost always essential. This is because the absorption spectra become very complex in regions of overlap between bands from different materials. Caspersson's work shows that the results are adequate if the equivalent slit-width is 2 mμ, which can be produced with prism [10, 11, 15, 21] or echellette grating [2, 14] monochromators.

The Basic Optics

A main component is the microscope, which has two functions; it forms a magnified image of the object, and also produces a high light flux through it, which is particularly important in ultra-violet and fluorescence microscopy, where energy is at a premium. The image quality is just as important in photometry as in visual observations. Aberrations alter the intensity distribution in the image plane and distort the photometer readings. These requirements define the demands on the optics, particularly as regards numerical aperture. The choice of condenser and objective aperture for cytophotometry has been dealt with in detail [2, 9, 20, 22–24], the important parameters being (1) resolving power, (2) image sharpness required for isolating small image areas, (3) diffraction effects at low apertures, (4) scattered light interference at high apertures, (5) variation of mean optical path in specimen with condenser aperture. Theory and practice indicate that the best apertures lie between 0·6 and 0·9.

In spectral studies the image must be sharp at all wavelengths used; this also applies to scanning methods, as preliminary focussing and choice of area are done in the visible (usually), and the focus must be unchanged on switching over to ultra-violet. Normal lens objectives cannot satisfy this requirement; to cover the comparatively narrow 330–220 mμ range Caspersson used two Kohler and Rohr objectives, while the 550–330 mμ range was covered with an apochromat + correcting lens [10]. Poor results are obtained if objectives are used at wavelengths other than those for which they were computed [22]. The only adequate solution is to use mirror systems, as these give sharp images throughout the spectrum; the use of these systems has been reviewed [3, 25–27]. They are widely used because they are achromatic, and because they have some other advantages in quantitative work. Mirror objectives give sharper images, because less unwanted internal reflections occur; the transmission varies little with wavelength, so wavelength scanning is more readily effected. The working distances are also long; a lens objective of 0·65 N.A. has a working distance of 0·5 mm, whereas a reflecting one works at 12–13 mm. Heated and cooled stages can then be inserted, accessory devices mounted, etc. A reflecting condenser is usually used as well.

Several types of reflecting microscope have been developed in recent years; the Burch microscope has been widely used for ultra-violet and infra-red work [16, 18, 28]. Burch-type objectives have two mirrors, one of which is aspheric (up to N.A. 0·65), or both (up to N.A. 0·95). The central obstruction is only 14 per cent; if auxiliary lenses and immersion are used the N.A. can be raised to 1·4. The interest

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in low-temperature studies led to the special Thornburg objective [29] being designed; this has one hyperbolic and two spherical mirrors. The N.A. is 0·77, magnification × 100, central obstruction 34 per cent of the diameter, working distance 7·5 mm.

The visual observation system in a cytospectrophotometer is important; an unstained object is of low contrast in the visible, so it is difficult to find the structures of interest and to set them in the photometer field. Luminescent screen methods are often used to examine the ultra-violet image; Caspersson used such a device, with an intense source (spark, capillary lamp). Preference is, however, usually given to viewing the preparation in the visible, in order to avoid long ultra-violet exposures; dark-field and phase-contrast methods are therefore often used. King [30] has described a phase-contrast device for the Burch microscope; in this the objective stop is imaged onto a plane where the phase plate can conveniently be sited. The same principle is used in Walker and Deeley's device [15].

Satisfactory contrast can be obtained with reflecting or combination objectives using a central stop [14], the illuminating cone of rays must have an angle slightly exceeding that of the central stop in the objective, in order to give adequate contrast in the scattered-light image.

Scanning Devices

These are used in sequential cytophotometry, and can be mechanical, optical or electronic; the siting and design of the scanner depend on the type of scan used. If the object itself is scanned the scanner can be on the microscope stage or be part of the illumination system. The detector photocathode almost always lies in a plane conjugate to the plane of the objective pupil.

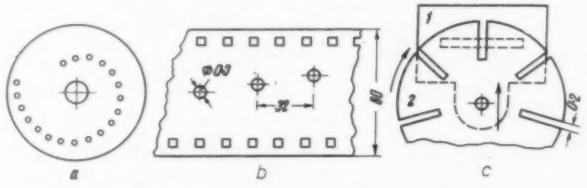

FIG. 1. Mechanical scanning devices.

A Nipkow disc is the simplest scanner (Fig. 1a); this has been used by Mellors and Silver [31] and by Loeser and Bercley [32], in studies on the fluorescence of stained cells and nuclei. The disc bears holes equally spaced along a spiral; when the disc rotates in the image plane the image is scanned along lines, equal in number to the number of holes. The angular spacings between the holes are such that only one hole appears in the field of view at any one time, so the light from one point in the image falls on the photocathode. The disc can be replaced by an endless film loop, as in Fig. 1b; Bourghardt *et al.* [12, 33] have used this type of device, with 120 holes, which divided a 36 × 36 mm field into 12,000 points, each point corresponding to an area of 3 μ^3 in the object plane. Fig. 1c shows a device due to Deeley [19], in which the scanning aperture is defined by the intersection between two narrow slits. The slotted plate 1 and the radially slotted disc 2 are mounted on a common axle and rotate clockwise at different rates.

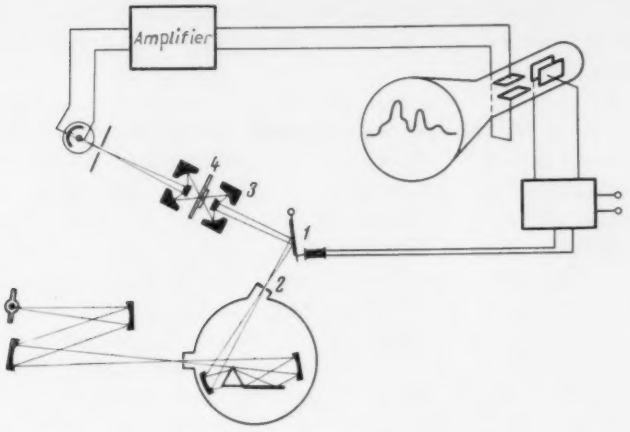

FIG. 2. Scheme of Exner's apparatus.

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Sequential cytophotometry is intended to avoid the errors associated with nonuniformities in the object; the nonuniformities in the various scan lines are often more or less the same, so in such cases the whole field need not be photometered. It is enough to know the optical density distributions along several parallel lines to derive the mean optical density; this is the basis for the one-line scanner, as in Thorell's device [13], in which the image is scanned over a diaphragm in front of the photomultiplier using a rocking prism. The same system is used in a television-type

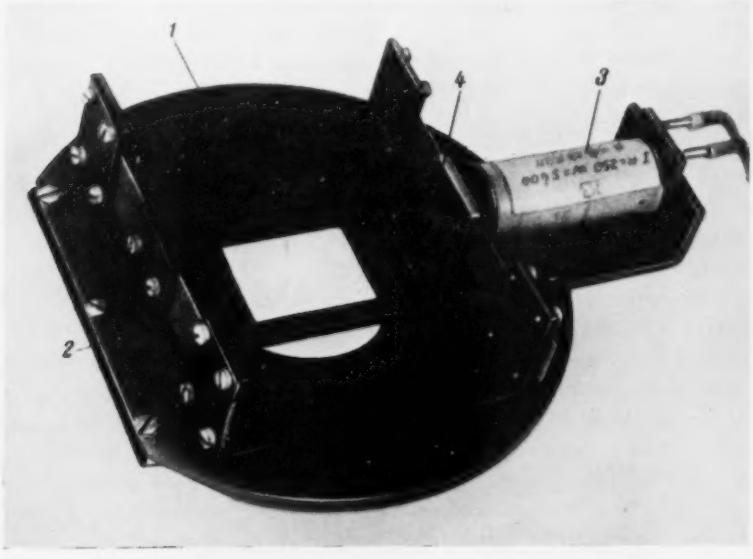

FIG. 3. Electromagnetic scanning device.

system [32] for absorption studies, in which the microscope image is projected onto a Vidicon or Orthicon tube, and one raster line is transferred to an oscillograph screen as an absorption curve.

If the scanner is on the image side of the microscope a large area of the preparation has to be illuminated throughout the measurements, which increases the scattered light and the danger of damage to the specimen; the microscope optics must also provide a high grade image over the whole field. The first two disadvantages are overcome in the flying-spot system; in this a very small spot is scanned over the specimen, and the transmitted intensity varies in accordance with the optical density. The advantages of the method are cogently demonstrated by Exner [34] and Loeser and Bercley [32] for living preparations. In Exner's apparatus (Fig. 2) the scanning is effected with a rocking mirror, driven electromagnetically; the exit slit on the monochromator 2, is imaged on the specimen 4, after reflection from the mirror. In Loeser's apparatus a raster on the screen of a short afterglow cathode-ray tube is imaged on the specimen; in this system the wavelength used depends on the screen coating. Recently Montgomery *et al.* [35] have produced a television-type microscope having a light-source tube with its maximum emission at 260 mμ and a band-width of 10 mμ; this device should prove of great value in ultra-violet microscopy. Flying-spot methods minimize specimen irradiation, but the projection system still has to work over a large field area.

A major improvement has been effected by Caspersson [8–10], by moving the specimen relative to a fixed small light spot; the object is fixed in a holder attached to two quartz plates which are driven by the membrane on a mercury-filled vessel. The mercury pressure is varied by a hydraulic system, and the specimen can thereby be moved smoothly through 60 μ by bending the plates. The construction is very rigid, motions being reproducible to 0·05 μ. Fig. 3 shows a similar device developed by Korolev and Agroskin [14]; the specimen-holder (1) is fastened to two flat phosphor-bronze springs (2), and moves horizontally. The electromagnet (3) is fed with a saw-tooth waveform, which causes the armature (4) (plus specimen) to move in a similar fashion. The scan range can be varied from 20 to 100 μ electrically.

Measuring Equipment

Normal photomultiplier circuits are used to measure light fluxes in cytophotometers (or less commonly photocell ones). Gorlich [34] has considered the various light detectors that might be used in this work. Special measuring heads, described below, are also used.

A special feature of cytophotometry is that the light fluxes are very low; the energy flux may be only 10^{-12} W at a permissible level of radiation passing through the specimen (equivalent to a few hundred photoelectrons per second). The circuits used at this level must be of low noise, e.g. as in Thirnburg's quantometer [36]. The quantometer is used to measure the visible and ultra-violet absorptions of microscopic objects with high accuracy; the apparatus consists of a reflecting microscope in which the image is projected on a double slit, the measured area falling on one slit and the reference area on the other. A rotating disc of special shape causes the two beams to fall alternately on a multiplier, cooled in liquid nitrogen to reduce dark current fluctuations. The transmission at any wavelength is measured

in terms of the output pulse rate; it is claimed that pulse counting minimizes errors from light source fluctuation, amplifier drift and photomultiplier voltage variation.

An important problem in quantitative cytochemistry is to obtain data on many cells, so that statistically valid conclusions can be drawn. This is the object of the automatic microphotometer [12, 33], designed according to the principles of information theory. The light from a point on the specimen passes through a hole in a perforated film (Fig. 1b) to the photomultiplier; the resulting signal is transformed to a train of pulses of number proportional to the light intensity. The pulses are recorded in a magnetic tape store; the time taken to scan 12,000 points is 4 min. When the measurements are complete the printing unit is switched in; this processes the magnetic tape and prints the transmission on paper in the same sequence as the measurements. A 45 × 45 cm print of the specimen is also prepared; every point on the image has then a number giving its absorptions.

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The concentration and total content of absorbing material in the cell can [1] be calculated from the optical densities, but these are not measured directly, being calculated from $D = \log I_0/I$ (I_0 = incident intensity, I = transmitted intensity). The photocurrent is proportional to the intensity, so D can be calculated automatically with a logarithmic circuit; various such circuits are known. One such is used in Deeley's microdensitometer [19]; here the voltage across the photomultiplier is proportional to log I, if the photocurrent is constant. Electronic logarithmic circuits have also been used by Sinsheimer [2, 4] and Keohane and Metcalf [18]. In Caspersson's equipment an electromechanical logarithmic device (developed by Lomakka [37–38] is used; the main part is a low-resistance potentiometer with many sliders. The resistance between sliders can follow any law, including logarithmic; a servo compares the input signal with that appearing between sliders, and produces another signal proportional to the logarithm of the input.

It is technically simple to determine the total amount of absorbing material in the cell, P, automatically when the thickness of the absorbing layer can be taken to equal the cell thickness. A simple calculation gives

$$P = C_m V$$

where C_m is the mean absorber concentration and V is the cell volume. The Lambert–Beer law gives the mean optical density as

$$D_m = \mu C_m d$$

where μ is the absorption coefficient and d is the layer thickness. Hence

$$P = D_m V/\mu d = D_m S/\mu$$

where S is the cell area, and D_m is given by

$$D_{\mathrm{m}} = \frac{1}{Ln} \sum_{l=1}^{n} \int_{0}^{L} D_l \, \mathrm{d}L$$

Here D_l is D at some point, L is the scan line length and n is the number of scan lines. Substituting for D_m in the expression for P, we have

$$P = \frac{S}{\mu Ln} \sum_{i=1}^{n} \int_{0}^{L} D_i \, \mathrm{d}L$$

To find the total amount of absorber we must therefore integrate the optical density curves and multiply the result by some constant dependent on the cell dimensions; both operations are easily done with an RC integrator [19, 37, 38]. If signal supplied to the capacitor is proportional to D_t the p.d. between its terminals will be proportional to the integral. The constant can be inserted via the electrical parameters.

Apparatus

There is at present no Soviet or foreign commercial cytospectrophotometer, hence one always uses apparatus built up from standard units; Fig. 4 shows the general form used: (1) is the illuminator, (2) a filter, (3) a microscope of normal design, (4) a stop (small hole), and (5) a photocell. This sort of system has been described by Swift [39], by Mellors and Glassman [23] and by others [11, 40]. For large-scale histo- and cyto-chemical work extremely complex equipments on which any such type of work can be done have been built up in some institutions. Some are described below.

Cytospectrophotometer, without Schwarzschild–Williger effect (Tokio University)* [21, 41]. Naora's main problem was to reduce the scattered light, since illuminating large areas of specimen while only photometering small ones gives much scattered light (Schwarzschild–Williger effect). The effect is particularly noticeable if the area photometered absorbs strongly, while adjacent areas do so weakly; the effect can be reduced by illuminating only the photometered area. Since the illuminated area must be 1–5 μ in diameter, normal condensers cannot be used as they are insufficiently corrected; Noara therefore used a high-magnification microscope (1) (Fig. 5) for illumination, with the light-path reversed. Using an N.A. of 1·25 with an oil-immersion objective, a hole of 2–10 mm diameter was projected on the object, diminished in size 2000 times. The light source was a high-pressure Hg arc, a double monochromator being used to separate the lines. The image given by the second microscope (2) could be examined visually, photographically or photo-electrically. A light-path from the light source to the photomultiplier was used to check the light source stability.

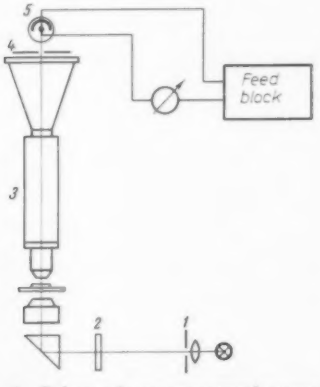

FIG. 4. Schematic cytospectrophotometer.

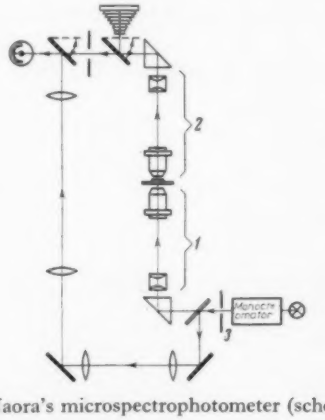

FIG. 5. Naora's microspectrophotometer (schematic).

* The names given are hereafter those given by the authors of the original papers.

Naora's conclusions on the scattered light were criticized by Thomson and Frazer [42], and by Ornstein and Pollister [24], who consider that the scattered light is reduced by reducing the condenser aperture, as well as by reducing the area illuminated. Since most cytophotometry is done at low and medium condenser apertures, the scattered light error does not exceed 3 per cent.

The probe cytospectrophotometer (*Vavilov State Optical Institute*) [14]. The local illumination method used here requires no special illumination system. The light falls on the specimen via the visual observation train (in part), as shown in Fig. 6. The output slit on the grating monochromator is replaced by a stop with a 0·03 mm

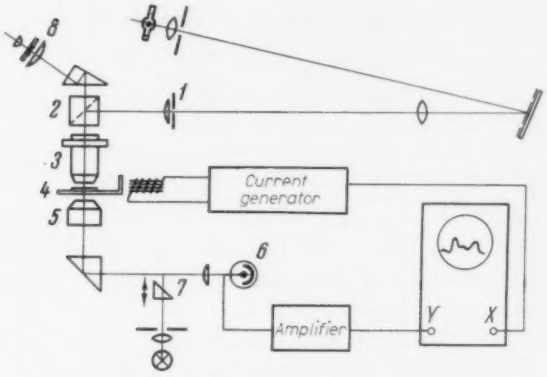

Fig. 6. The probe cytospectrophotometer (schematic).

diameter hole. This hole is projected onto the specimen using the semireflecting prism (2) and the objective (3). The light, focussed by the objective into a "light probe", then passes through the condenser (5) to the photomultiplier (6). The diffraction at the × 65 N.A. 0·8 reflecting objective gives a spot about 1 μ in diameter. The structures are observed and set in the probe in the visible, an extra illuminating system being used, with the prism (7) pushed in. The specimen is seen in the field of view of the micrometer eyepiece (8), in which the intersection of the cross-wires is conjugate with the light probe. The accuracy of absorption measurements is $\pm$ 2 per cent (or better) over the 230–600 mμ range. The apparatus is fitted with an electromagnetic device for moving the specimen, an amplifier, and an oscillograph to record the curves.

The recording microspectrophotometer (*King's College, London*) [15]. This automatically records the absorption spectra of cellular structures in the visible and ultra-violet; it can also be used for scanning at a fixed wavelength. The density range covered is 0–1, the spectral purity 2 mμ. The optical section (Fig. 7) consists of a double-beam measuring system (1–13) and (1–9; 14–19; 13), and a phase-contrast device for observing the specimen (22–28). The light source used in spectrophotometry is the xenon lamp (1), which illuminates the output slit (9) of a prism monochromator. The quartz prism (14), placed behind the slit, oscillates at 50 c/s, so the rays alternately fall on the receiver (13) either via the specimen in the microscope (10–12) or via the comparison path (15–19). The optical balancing device

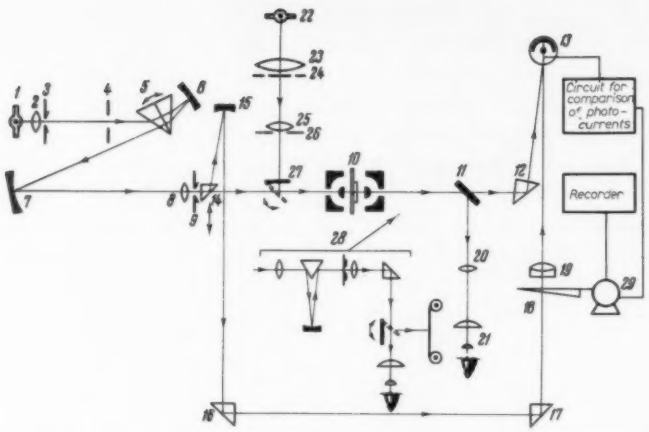

FIG. 7. Deeley and Walker's recording microspectrophotometer (schematic).

(a slit of variable width) balances the energy in the two beams. The servomotor (29) keeps the slit at the balance position; the slit is shaped so that the energy passed varies logarithmically with displacement, and the displacement recorded is proportional to the optical density.

Walker and Deeley, the designers, consider that the accuracy then depends only on the optical and mechanical devices, and is independent of the electrical circuits.

The visual observations employ the Hg lamp (22) and a phase-contrast device; the specimen can also be photographed. The reflexion diaphragm (11) restricts the area photometered, and enables one to observe the specimen during the operation. It is pointed out that the optical paths in the measurement and comparison beam are different; a systematic error is introduced into the density by the differing losses in the components. When reflecting microscopes are used the error can be reduced to 0·15 optical density units.

Recording microspectrophotometer (*MIT, USA*). This was designed by Sinsheimer and has been described [2, 4, 43] as being of high accuracy. The optical section (Fig. 8) is of normal type; the light from a hydrogen lamp passes through a grating monochromator and reflecting condenser to the specimen. The reflecting objective projects an image of the specimen onto the receptor diaphragm, which delimits the area studied. Low light fluxes are measured accurate to 1 per cent by a special electrical system which eliminates effects arising from source instability and photomultiplier voltage variations. Double-beam methods are used, the two beams being modulated at different frequencies (90 and 900 c/s) and falling simultaneously on the multiplier. The absorption curve is recorded in optical density terms, the spectrum of a clear area in the specimen being recorded first. The wedge (2) balances out light losses in the microscope path.

Universal ultramicrospectrograph (*Nobel Institute, Stockholm*) [8–10, 44–47]. This was built in Caspersson's laboratory and is used to determine the total amount of absorber in a cell. Absorption spectra of particular points can be taken, polarized

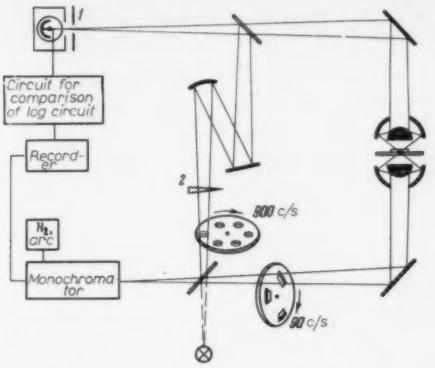

FIG. 8. Sinsheimer's recording microspectrophotometer (schematic).

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light used, etc. The general system used is shown in Fig. 9; a novel point is the automatic mercury micromanipulator (1), described above. When this mechanism operates the image is displaced relative to the receptor diaphragm (3); seen in the plane of the specimen, this stop has an area of 0·4 μ^2. The optical device (2) (a rocking plate) in the microscope tube is used to displace the image in a direction perpendicular to the first.

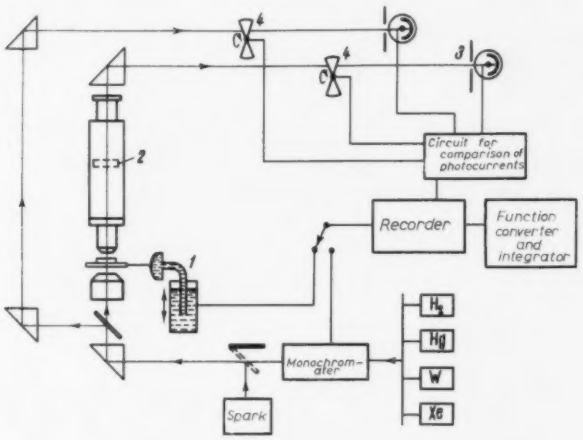

FIG. 9. Caspersson's ultramicrospectrograph (schematic).

The system is a balanced double-beam one; balancing is effected with rotating sectors (4), the angles of which can be adjusted during rotation. The error in absorption measurements at wavelengths down to 260 mμ is not more than 2 per cent during line scans, even if the source output varies by 20 per cent; at shorter wavelengths the error is somewhat greater. The Lomakka functional converter and integrator (see above) receives the output signal and continuously calculates the total amount of absorbing material in the area covered. Many cells can thus be covered in a short time.

Caspersson and others have claimed that it is necessary to measure the absorptions of microstructures very accurately; as regards recording absorption curves this is not disputed; but it is doubtful if accurate measurements of the total amount of absorbing material are required [1, 20]. Caspersson's critics consider that there is here no need for the apparatus to be able to measure to a fraction of a per cent, since the error in the final result is mainly biological in origin, and may be as high as 10–15 per cent. If excessive accuracy is demanded the apparatus becomes unnecessarily complex.

SUMMARY

Microspectrophotometry is the modern method of quantitative cyto- and histochemical analysis; either the selective absorption by the substance studied, or the absorption of a dye bonded to it by an appropriate reaction, are used. The absorption can be recorded objectively either photographically or photoelectrically. Photoelectric systems have become common in recent years, in cytospectrophotometers in which microscope and spectrophotometer are combined. Existing cytospectrophotometers were developed in scientific institutes using modern optics, electronics and automatics. The chemical compositions of individual cellular structures 1 μ in size have been established quantitatively. Cytospectrophotometers can also be used for studying solutions, films, microcrystals, etc. The accuracy of absorption measurements is 1–2 per cent.

Translated by J. E. S. BRADLEY

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METHODS OF EXPERIMENTALLY IRRADIATING SMALL ANIMALS WITH CENTIMETRE WAVES*

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RADIO waves in the centimetre range have attracted the attention of medical and biological workers in recent years; these wavelengths have been widely studied for their use in physiotheraphy, and also for any damaging effects they may have on man. More than 150 papers have appeared, including reviews [1–4].

One of the basic difficulties in animal experiments in this field is that special methods and apparatus for dosimetry have had to be developed, and so far no adequate solutions have been found. The methods used abroad are considered, and some of our work is described, in this paper. The methods used for experimental studies fall roughly into three groups, by dosimetry: (1) relative dosimetry; (2) approximate dosimetry; (3) fairly exact dosimetry.

1. *Methods Using Approximate Dosimetry*

The Raytheon Microtherm apparatus for physiotherapy has been used in most work; this is a magnetron generator (c.w.) operating at 2400–2500 Mc/s ($\sim$12 cm), of maximum power output 125 W. The power is fed through a coaxial cable to the radiator, consisting of a dipole plus reflector; three types of radiator are used. Types *A* and *B* have hemispherical reflectors (10·2 and 15·2 cm in diameter) and *C* conical ones. Dosages are altered by adjusting the output power, irradiation time and distance from radiator to object; the shape and size of the irradiated surface, and the intensity distribution over the surface, have been measured by thermal methods

* Biofizika **3:** No. 3, 354–358, 1958 [Reprint Order No. BIO 152].

[5]. Fig. 1 shows diagrams of intensity distributions derived by measuring the temperature rise at points on the irradiated surfaces. Type *C* clearly gives the maximum intensity at the centre of an elliptical surface, while *A* and *B* give their maximum intensities at the periphery of a circular surface.

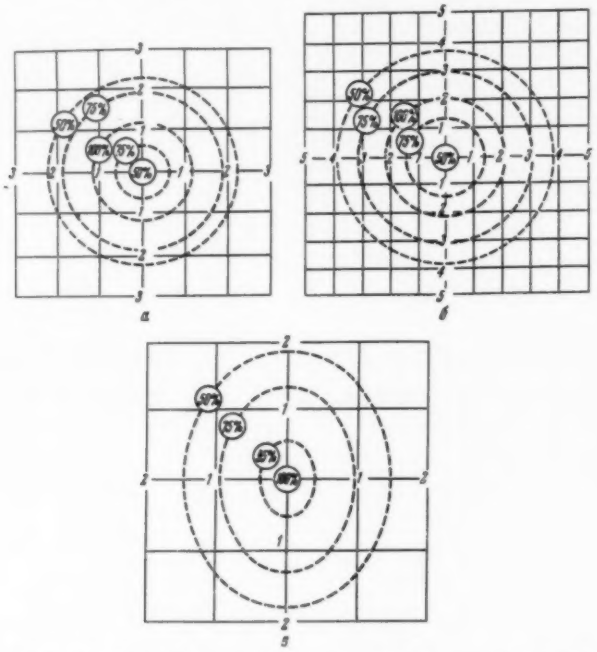

FIG. 1. Intensity distributions in centimetre wave irradiations. (a) Radiator type *A*, 2·5 cm from object; (b) radiator type *B*, 5·08 cm away; (c) radiator type *C*, 5·08 cm away.

2. *Units Used in Absolute Dosimetry*

The dimensions of the animals used are comparable with the wavelength, while the absorbed energy ultimately depends on the wave intensity at the surface. If this latter is evaluated we get the dose rate p, say in mW/cm² or W/cm². The radiation intensity can be directly measured with some appropriate instrument, but in animal experiments it is the absorbed dose that is important, i.e. the effective intensity K. Part of the incident energy is reflected, part absorbed in the fur, and part in the surface tissues.

The reflection coefficient ρ (ratio of reflected and incident energies) depends on the complex dielectric constant $\epsilon = \epsilon' - j\epsilon''$* which varies with λ; ρ can be calculated [2, 6] roughly from

$$\rho = [(\epsilon' - j\epsilon'')^{\frac{1}{2}} - 1]^4/(\epsilon' - j\epsilon'' - 1)^2. \qquad (1)$$

Fairly accurate measurements of ϵ' and ϵ'' have been made for human tissues [7, 8], and for the tissues of certain animals [9].

Theory shows that ρ must also depend on the thickness of the subcutaneous fat layer [2] in the centimetre and decimetre range of wavelengths; in rats, rabbits and

* Other quantities can be used (which are related to $\bar{\epsilon}$), e.g. the dielectric constant ϵ, and the conductivity σ, or the propagation and absorption coefficients, ρ and α.

dogs $\rho \sim 60$ per cent [11] for 10 cm waves, which differs little from the calculated value. Hence in practice K can be determined from

$$K = p(1-\rho) \tag{2}$$

where p is measured and ρ calculated.

3. *Approximate Dosimetry*

Approximate dosimetry can be realized by using a horn placed some distance away and measuring the intensity at the irradiation site. We used this system with white rats at 10 and 3 cm wavelengths. The rats were placed in the cells of a chamber made up from polystyrene sheet* pierced with holes to allow air to circulate. The rats were about 4 m from the conical horn (25 cm diameter 50 cm long) with the centre of the front end of the chamber on the horn axis. An IPP-10 unit [10] was used to measure the wave intensity; a receiving horn was put in place of the chamber in such a way that the end of the horn replaced the front end of the chamber. The intensities at the centre and at the edges of the chamber were found to be virtually identical.

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In the irradiations the chamber was placed as above, but 1 m from a horn of rectangular section (9 × 13 cm, 15·5 cm long). Measurements with a 31-IM showed intensities at the edges some 20–25 per cent lower than at the centre. To produce identical conditions the rats were moved about between cells during repeat measurements. The zones irradiated with the 3 and 10 cm waves were delimited using screens coated with a special material which absorbed the waves. This method is convenient for use with a set of small animals.

A similar method has [11] been used for irradiating cats, rabbits and dogs with 10 cm waves. The animals were freely suspended (the rabbits and rats in cages, the dogs in canvas slings) at a defined distance from the horn. A Hewlett-Packard 430 c unit was used to measure the intensity, using a directional horn and an attenuator. The irradiated region was delimited with sheets of absorbing material.

4. *Comparatively Accurate Dosimetry*

In 1950 a method by which very accurate dosimetry could be effected was proposed [12]. The arms of subjects were irradiated by direct contact with the horn, which was fitted with a special matching device to ensure that all the energy entering the horn was absorbed. We have used this method on white rats; Fig. 2 shows a simplified diagram of the wave-guide system used which contained a generator (1), an attenuator (2), a directional section, which tapped off a definite small raction of the energy and fed it to the power meter (4), the matching device (6), the measuring line (5), with which the degree of matching could be checked, the curved guide (7), which passed the waves to the horn (8), and the irradiation chamber section (9).

In the simplest mode $(H)_{01}$ the power is unevenly distributed across the section; along the electric field direction (parallel to the short side of the guide) the power density is constant, whereas normal to this direction the density decreases from the centre outwards. The intensity at the sides of the animal was calculated to be 90 per cent of that at the centre.

* Polystyrene hardly reflects or absorbs centimetre waves.

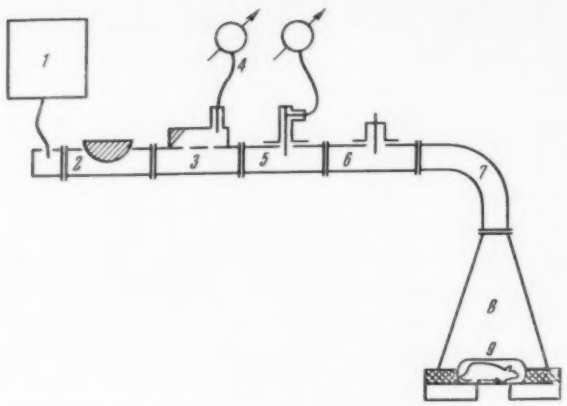

FIG. 2. Simplified diagram of the wave-guide dosimeter for irradiating rats in a horn.

The dosimetric section was calibrated by measuring the wave power (with a power measuring instrument replacing the horn), about 0·001 of the total power being fed to the power indicator (4). The next stage was to adjust the matching unit; the irradiation head was fixed on (with the animal in) and the matching unit adjusted to give the lowest standing-wave ratio at a low power level; reflection from the object is then maximally compensated. Standing-wave ratios of 0·1–0·2 were easily produced; then not less than 99 per cent of the power is absorbed in the animal or in the surrounding absorber. The changes occurring on using an animal of somewhat different size were very slight, so the matching unit was only adjusted from time to time.

The irradiation procedure was very simple: the irradiation cell plus animal was fitted up, then the attenuator and power indicator were set to give the required power level. Many tests have shown the method to be extremely convenient and to give quite accurate dosimetry,

Each of the three methods has its advantages. The Microtherm method is good when small areas are to be irradiated and relative dosimetry suffices. The second method is convenient when several small animals are to be irradiated together; approximate dosimetry is possible. The third is most suitable when the dosimetry must be comparatively accurate.

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THE IMPORTANCE OF CERTAIN POINTS OF METHOD IN THE LOCAL RESPONSE OF TISSUES TO CENTIMETRE WAVES*

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THE present work was done in connection with approval tests on a prototype centimetre wave generator for medical use (CWM) produced by the Vologdin High-Frequency Current Institute. The work was done using a UVCh-31 pulse magnetron (pulse repetition frequency 1000/sec pulse length 1 μsec, $\lambda = 3$ cm). The power was transmitted via a circular waveguide and a conical horn 6 cm in diameter to the object. Except for one series, irradiations were done with a separation of 10 cm between the horn and the surface irradiated. The radiation intensity was determined from the energy flux/cm^2 of irradiated surface. The total energy supplied was calculated from special tables and was recorded by a high-frequency wattmeter. The maximum generator output was 200 W.

We studied skin heating and vascular reactions in man following local irradiation as functions of (1) radiation flux, (2) air gap between horn and surface, (3) irradiation time. The vascular reactions were determined by skin thermometry; Shargorodskii [1], Mishchuk [2] and Minut-Sorokhtina [3] have shown that the method is adequate. Healthy normals (colleagues, students) were used, of age 20–45 years, in the morning at an air temperature in the laboratory of 18–20°C and at normal humidity. The middle joint of the forefinger was used; the temperature was measured in the centre of the irradiated area, where the horn cone gave the highest intensity [4]. The subjects were in the laboratory with ungloved hand for 30 min prior to the tests. The temperature was measured with a Mishchuk-type apparatus, the PK-5, serial No 5-41121 (accuracy of measurement $\pm$ 0·1°), immediately prior to irradiation, immediately after shut-down, and then every 2–5 min until the original level was reached. In all, 82 subjects were used.

1. *Dependence of Skin Heating and Vascular Reaction on the W/cm^2 in the Beam*

The instructions with the UVCh-31 state that when the horn is in contact with the surface a flux of 0·36 W/cm^2 is the greatest flux which does not excite any sensation in a healthy individual over a prolonged period. We therefore began our studies at 10 cm distance with a flux of 0·36 W/cm^2, proceeding from there to 0·56, 1·5 and 3·5 W/cm^2, which correspond to 1, 1·5, 4 and 10 doses.† In the first three series the tests lasted 10 min, in the last until the heat became painful. The results are given in Table 1.

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† These doses have been rounded off, for convenience in practical use.

TABLE 1. VARIATION OF HEATING AND VASCULAR RESPONSE WITH FLUX OF ELECTROMAGNETIC ENERGY

Series No.	No. of subjects	Radiation flux (W/cm^2)	Total dose (W)	Subjective reaction	Time lag (min)	Skin erythema	Skin temperature rise (°C)	Time for temperature to return to original value (min)
1	10	0·36	13	Slight heat	10	Slight, rapidly vanishing	1·5	8
2	12	0·56	20	Medium heat	10	Medium, longer lasting	5·3	14·5
3	12	1·5	50	Strong heat	Burning sensation in 4 subjects after 4–5 min	Acute, prolonged	8·0	67
4	10	3·6	130	Very strong heat going over to burning	0·5–1·2	Very acute, prolonged	9·2	Not measured

It is clear that, as the power density increases, (1) the skin heating increases, (2) the after-effect duration increases very rapidly, (3) the subjective sensation becomes stronger, and (4) the maximum permissible irradiation time decreases. An increase from 1·5 to 3·6 W/cm² reduced the time usable by a factor almost of 10 (the usable time does not exceed 30–70 sec at 3·6 W/cm²). In view of the danger of burns, this power should not be used in practice. Cook's data [4] indicate that at $\lambda = 10$ cm and 0·6–0·8 W/cm² over an area of 140 cm², pain is experienced at a skin temperature of 45 °C. Our data indicate large individual variations in the skin temperature giving a painful sensation, the range being 39·5–44·5 °C in our subjects.

The temperature rise in any object is determined by the balance between absorbed power and rate of heat loss; heat is lost by convection, radiation, evaporation, conduction, and blood circulation in a tissue. The effects of centimetre waves are largely determined by air conditions (temperature and humidity) and by the response of physiological mechanisms responsible for protecting the tissues from heating up. To determine how the vascular system reacts during the time the radiation is applied we measured the skin temperature on the other arm, as being equivalent [6, 7]. We also did four series of tests (with 10 subjects in each) at the same power horn distance and irradiation time, in which the temperature was measured at corresponding points on the non-irradiated hand.

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Results. At 0·36 W/cm² the skin temperature was unaffected; at 0·56 W/cm² it rose by 0·2–0·4 °C; at 1·5 W/cm² the reaction had two stages: a fall of 0·2–0·5 °C in the first 1–2 min, followed by a rise of 0·6–1·4 °C. At 3·6 W/cm² a fall in temperature of 0·5–0·9 °C was found, but measurements were not continued as the sensations became painful. The vessels were found to react differently as the power was raised; at medium powers the vessels dilated, whereas at higher powers we got the above two stages, though the second stage was not observed at very high powers because the test was stopped in the first stage. The vascular reaction began to take an unfavourable turn at 3·6 W/cm², since vascular contraction caused ischaemia and favoured still greater heating. Worden *et al.* [7] have also observed this on irradiating the extremities of dogs under conditions of complete ischaemia, when much heating occurred. The amount of heat liberated in less than a minute in the tissue exceeded the value which could be balanced out by physiological processes.

Our data also show that, although the powers used did not affect the body temperature, respiration, or pulse, temperature measurements on distal areas showed a distinct rise in skin temperature, indicating a vasculomotor reflex of nervous origin to local irradiation. This agrees with Rogov's data [6] which were derived by plethysmography. By analogy with other physiotherapy effects, we may assume that this reaction to centimetre-wave irradiation is of nervous reflex origin.

2. *Variation of Heating and Vascular Reaction with the Air Gap Between Horn and Irradiated Surface*

The wave energy is most completely absorbed, and therefore the heating greatest, when the horn is in direct contact with the surface [4, 8]. Heat loss by convection, radiation, and evaporation, is then excluded, and only blood flow and conduction can act. We consider direct attachment unsuitable for medical purposes, since the state of the surface cannot be checked visually, and the horn has to be sterilized if

the apparatus is used in diseases where the skin is not intact. In choosing a suitable air gap we were guided by the fact that, although many have used a 5 cm gap, Worden *et al.* [7] have found that burns occur if the blood circulation is hindered in 15 and 20 min irradiations. Since the local circulation is often disturbed in pathological conditions, and 20 or 30 min irradiations are commonly used in medical practice [9, 10], we used a gap of 10 cm. To establish the effect of the gap we did two series of tests at 0·56 W/cm² with 10 exposures, at gaps of 10 cm and 3 cm respectively.

Results (mean of 10 in each series). In series 1 the skin was heated up by 5·3 °C, and returned to normal after 14·5 min; in series 2 the rise was 6·3 °C, the return taking 28·1 min. The greater rise and slower fall at the smaller gap undoubtedly indicate impaired heat loss, so external heat losses must play a considerable part. This is confirmed by data of Daily *et al.* [11] on eye irradiations in animals; at identical power densities and exposures a reduction from 7·62 to 5 cm in the gap between horn and eye caused the cornea to become opaque. Thus if the air gap is reduced there is danger of physiologically unacceptable heating at high powers, particularly if vascular reaction is impaired.

3. *Variation of Heating and Vascular Reaction with the Irradiation Time*

Three series of tests were done, with 10 subjects in each. The power density was 0·56 W/cm², the air gap was 10 cm, exposure in series 1 was 10 min, in series 2 was 20 min. In the third series the exposure was also 20 min, but the generator was switched off at 2, 5, 10 and 20 min, and the skin temperature was measured.

Results. In series 1 the skin temperature rose by 5·3 °C, and returned to normal after 14·5 min. In series 2 the skin temperature rose by 5·5 °C, and returned to normal in 25·4 min.

The difference between the 10 and 20 min exposures is small, as we had previously found for VHF treatments [12]. Increasing the exposure time had a greater after-effect, which last nearly twice as long for the 20 min exposure; this topic demands further study. It may be that more tissue is heated up in 20 min, so cooling takes longer; long irradiations may also result in venous hyperaemia.

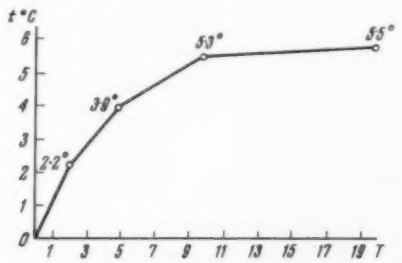

FIG. 1. Heating of a healthy human forearm (mean of 10 measurements). Ordinate: Rise in skin temperature; the origin is taken at the initial forearm skin temperature.

In series 3 we studied the rate of skin temperature rise; as the centimetre waves might alter the thermocouple reading, the generator was switched off, as noted above. Fig. 1 gives the results; the rate of rise was clearly greatest in the first 2 min, the rate

falling off in inverse relation to the time. The temperature rise thus followed the asymptotic laws for normal heat flow conditions in physical systems, at least in a general way, though the general physical character was largely regulated by the physiological heat loss mechanisms; Presman [13], for instance, has shown that dead

TABLE 2. DOSIMETRY TABLE

Dose	Total power (W)	Power density (W/cm^2)	Irradiation time (min)
Low	13	0·36	10–20
Medium	20	0·56	10–20
High	50	1·5	Up to 10

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tissues heat up more than living ones. Our results can be generalized to give the practical table of doses shown in Table 2 (using the condition described above). In this way only wattmeter readings are required to set up appropriate centimetre-wave dose conditions.

SUMMARY

(1) The skin heating and vascular reaction produced by centimetre waves depend on the power density, the exposure time, and the air gap between horn and irradiated surface.

(2) At low and medium powers the vessels on the opposite extremity (not irradiated) dilate, whereas at high powers we get a brief contraction followed by a prolonged dilation. The vascular reaction on the non-irradiated extremity probably corresponds to the rate of temperature rise on the irradiated surface.

(3) When the air gap is reduced at fixed power density and exposure time the tissues are heated more strongly, and the after-effect lasts longer because heat loss is hindered.

(4) Raising the exposure time to 20 min (at fixed power density and air gap) causes a relatively slight increase in skin temperature, but greatly extends the duration of the after-effect.

(5) The dose system proposed for practical use enables one to set up appropriate centimetre-wave dose conditions using only wattmeter readings.

Translated by J. E. S. BRADLEY

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METHODS OF DRYING PREPARATIONS FOR FLUORESCENCE MICROSCOPY*

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FLUORESCENCE microscopy has become widely used in recent years, and many workers have pointed out its advantages in histological and cytological studies [1–5]. So far the main obstacle to its wide use has been the lack of suitable embedding media; Canada balsam and other such materials usually employed are unsuitable. Special non-fluorescent media have recently been proposed for the purpose, namely, vinylin (polyvinyl alcohol in water) [6], polystyrene [7] and acrylate resin [8, 9]; with these one can prepare durable fluorescent specimens. Another serious obstacle has so far been the lack of convenient methods of drying sections before embedding. The methods normally used are air-drying in an oven, or spirit washing, but the sections curl up and the cells are greatly deformed; if sections treated with fluorochromes are spirit-washed the dye is almost entirely leached out.

Our studies indicate that freeze-drying at low temperatures can be used to advantage; in this way metabolism or chemical reactions are stopped at once, and even diffusion is greatly impeded [10]. An apparatus built at the Institute of Biophysics for use with pieces of tissue about 1 mm thick (see Fig. 2 of [10]) was used, and the following method was developed.

(1) Sections 6–8 μ thick are cut from tissues fixed in neutral 10 per cent formalin; the sections are frozen for cutting and removed in distilled water.

(2) The sections are placed in 25 per cent ammonia for 8–10 min.

(3) This is followed by a wash in water for 1 min.

(4) Acridine orange, 1 : 1000 in distilled water (1–1½ min), is used for staining.

(5) Section again washed in water for 1 min.

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(6) Treatment with a 10% solution of aluminium sulphate for 3–4 min follows.
(7) Section again washed in water for 1 min.

The sections are placed on cover glasses and the excess water removed with filter paper; the cover glass is then transferred to *iso*octane cooled by immersing the tube containing it in a dewar containing liquid nitrogen until a layer of solidified *iso*octane 2–3 mm thick forms inside the tube. The still liquid *iso*octane is then at about −130°C. The rapid cooling produces ice crystals so small as to be at the limit of resolution of the microscope, and the structure is not appreciably deformed.

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FIG. 1. Section of normal rat kidney dried in the vacuum equipment. Stained with acridine orange. Objective × 40, eyepiece × 5.

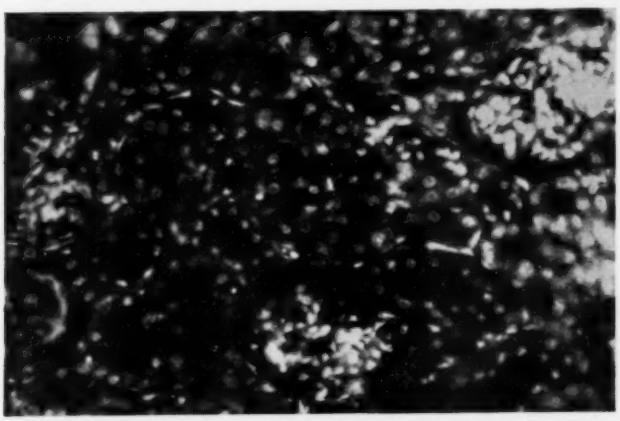

FIG. 2. Section of normal rat kidney dried in alconol. Stained with acridine orange. Objective × 40, eyepiece × 5.

The frozen sections are placed in the tissue holder in the freeze-dryer, also per-cooled with liquid nitrogen. After starting the rotary pump the vacuum is kept at about 10^{-3} mm Hg, with a temperature of about −170°C. With thin sections the additional diffusion pump is not required. The temperature is slowly raised using an electric heater fitted to the tissue holder; the water sublimes off, leaving the cells undistorted. The specimens are kept under vacuum until they are somewhat above

room temperature (25–30°C) is reached, to prevent water vapour condensing from the air on removal. The drying time is 15–20 min. More prolonged drying causes the section to break up. The very fragile dry section is transferred to a slide with a needle, and coated with 1–2 drops of polystyrene solution, and covered with a cover glass.

We used this method to prepare reference specimens from various human and animal tissues (Fig. 1); the specimens usually stored very satisfactorily and gave good fluorescence. The nuclear structures were retained and fluoresced yellow-orange, while the protoplasm fluoresced dull green. The method was in every way satisfactory.

We also tested the spirit-drying method; to prevent the fluorochrome being washed out we replaced the aqueous acridine orange by a mixture containing

distilled water	10·2 parts
acridine orange	0·02 parts
absolute alcohol	1·0 parts
glycerol	1·0 parts.

Other fluorochromes can also be added.

Method of Preparing Specimens

(1) The sections taken from the freezing microtome are stained in the above solution for 0·5 min.

(2) The sections were washed in distilled water for 1–2 min.

(3) They were then dried in 96° spirit, using 3 successive 1 min washes.

(4) The sections were clarified in phenol–xylene for 0·5–1 min.

(5) They were finally mounted in polystyrene under cover glasses.

The specimens are usuable immediately after preparation, and the fluorescence is bright, and similar to that found on vacuum drying; no change occurred on storage (Fig. 2).

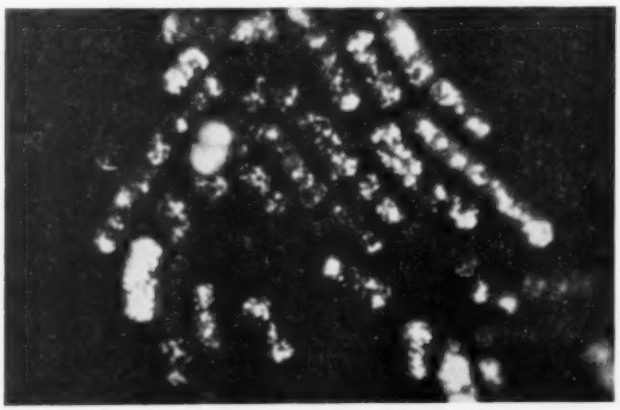

FIG. 3. *Endomyces magnusii* cells. Fat and lipids stained with Nile blue. Objective ×40, eyepiece × 5.

We also used plastics for embedding; when the stained preparations were air-dried, or dried over a flame, they could be embedded immediately after preparation in polystyrene. To preserve cells without prior drying we used polyvinyl alcohol in

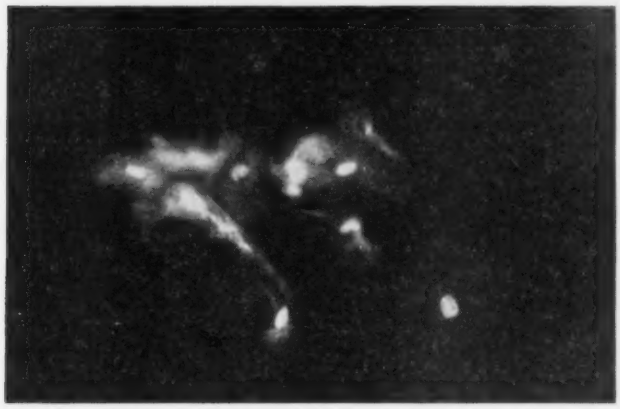

FIG. 4. Epithelial cells from a human oval cavity. Stained with acridine orange 1 : 10^4. Objective × 40, eyepiece × 5.

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water (vinylin). The preparation (cell suspension) was prepared as a smear on a glass slide and a few drops of polyvinyl alcohol solution added. On drying, the vinylin formed a thin film which trapped the cells. The surface of the film was smooth enough to allow of high magnifications. In these preparations the fluorescence was bright and the cell structure well retained (Figs. 3 and 4).

The methods described here are convenient for preparing good, stable specimens for fluorescence microscopy, and should be of some considerable value in histology and cytology.

We are indebted to Professor M. N. Meisel' for valuable advice.

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FLUORESCENCE MICROSCOPY STUDIES OF THE EARLY CHANGES INDUCED IN RAT BONE-MARROW BY CHLOROETHYLAMINES*

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The anti-tumour agents at present available (chloroethylamines, ethylenimines, etc.), plus ionizing radiations, greatly depress the activity of the haemopoietic system, but studies of the early changes they induce in bone-marrow is difficult because of the lack of adequately sensitive methods. Recently Meisel' *et al.* [1] have developed a new method, in which the cells are treated with a fluorescent dye containing an agent which quenches the fluorescence of undamaged cells; this renders the damaged cells more readily seen. In this way fairly early changes in the bone-marrows of X-irradiated animals have been demonstrated [1]. Chloroethylamines and X-rays have very similar effects [2–5], so we used fluorescence microscopy in studies with chloroethylamines (sarcolysin, dopan, embichin) and X-rays (for comparison).

MATERIALS AND METHODS

White rats of uniform age and weight were used; the sarcolysin and embichin were injected intraperitoneally in saline. The dopan, being insoluble, was administered *per os*, suspended in starch paste. The marrow was taken from the tibia; 1 mg was weighed out on an analytical balance and mixed with a drop of the fluorochrome solution on a slide. Three preparations were made from each marrow; one was stained with an acridine orange–acid fuchsin mixture, the second with the same mixture plus Congo red (the latter used as quenching agent), while the third was fixed in methanol and stained (Romanovsky–Giemsa). The solutions were prepared by mixing 1 : 1000 solutions of the ingredients. The first mixture was prepared from 0·1 ml of acridine orange, one drop of acid fuchsin, plus 5 ml of Ringer; the second was the same, except that 0·2 ml of Congo red solution was added. The treatment lasted 2–4 min, and was done in a humidified chamber; the preparation was then covered with a cover glass and the excess liquid removed with filter paper. The preparations were sealed round the edges with paraffin wax, to prevent evaporation. The exciting radiation used was blue (visible).

RESULTS

Early changes in bone-marrow nucleoprotein produced by chloroethylamines can be detected by vital staining with acridine orange plus Congo red [6, 7]. The bone-marrow cells can be differentiated in this way, but not as precisely as with

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normal haematological stains; differentiation between young granulocyte forms is particularly difficult. Promyelocytes cannot always be differentiated from myelocytes, and myelocytes can only be differentiated from metamyelocytes by the shape and structure of the nuclei, or by comparing nucleus and cytoplasm. The granularity of granulocytes is not always shown up with acridine orange, which also does not show up the chemical nature of the granules completely (whether basophilic, azurophilic, or neutrophilic), as all cytoplasm granules fluoresce red. As the young granulocytes were difficult to differentiate, we combined them all under the general name of young granulocytes. These granulocytes had nuclei with fine granular structures which fluoresced green; the cytoplasm fluoresced orange or red and contained red inclusions of various sizes. The adult granulocytes (with segmented and rod-like nuclei) were readily differentiated, as were the adult agranulocytes (lymphocytes, monocytes). The nuclei of lymphocytes, monocytes and leucocytes fluoresced bright green of various shades (with some cells darker or lighter than others). The cytoplasm of leucocytes usually fluoresced orange, or more rarely red, with red granules; lymphocytes and monocytes had cytoplasm which fluoresced green or orange. The megakaryocytes, with nuclei giving a characteristic yellowish-green colour, and cytoplasm either greenish or rose-coloured, were readily differentiated. Plasma cells were distinguished by their intense rose-coloured cytoplasm fluorescence.

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Young erythroid cells were more readily differentiated than young granulocytes. Erythroblasts stages one, two and three were readily differentiated; they had rounded nuclei fluorescing bright green, and red cytoplasm, and could only be differentiated by size. Their fluorescence is comparable with those of lymphoblasts and lymphocytes, and so are the nuclei and cytoplasm; the three types are therefore sometimes difficult to differentiate. Reticulocytes in the peripheral blood are particularly readily identified, as they emit a greyish-red light and frequently contain reticular substance as small grains with bright red emission.

When rats are given sarcolysin at 5, 15 and 30 mg/kg, very bright cells can be seen in the bone-marrow within an hour or so; they are present in clusters. The numbers of such clusters increase rapidly at 2–5 hr, and then fall equally rapidly to almost zero. The numbers also depend on the dose (see Fig. 1). When the other two chloroethylamines are used at equivalent doses the effects are much less marked. Fig. 1 shows how the numbers of clusters vary over 24 hr with dopan (0·75, 1·5 and 3 mg/kg) and embichin (0·4, 1·5 and 2 mg/kg). Large doses are required to produce many damaged cells, which subsequently break up. With sarcolysin the damaged cells are apparent for a longer time (to 20 hr) than with the other compounds (12–15 hr). The damaged cells are only revealed if acridine orange plus fuchsin is used, but clearer differentiation is obtained by using the quenching agent as well; the nuclei of cells from control animals emit a diffuse green light, against a dark background. These cells are usually isolated ones, however, and the emission is relatively weak.

The species most sensitive to the chloroethylamines are of some interest; to this end we used the mixture containing quencher, and removed the excess liquid without covering the preparation with a cover glass. If groups of brightly emitting cells were seen, the preparation was fixed by applying methanol for 1–3 min. After fixation the preparation was dried and stained (Romanovsky–Giemsa) and studied in the

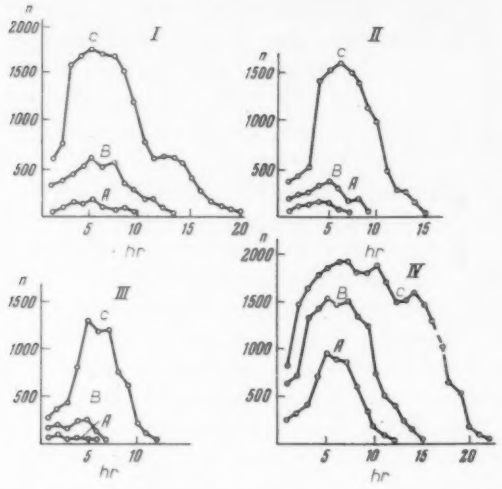

FIG. 1. Changes in rat bone-marrow induced by chloroethylamines and X-rays. Ordinate: no. of damaged cells, *n*. Abscissa: time after administration, hours.

(I) sarcolysin,	A = 5 mg/kg,	B = 15 mg/kg,	C = 30 mg/kg;
(II) dopan,	A = 0·75 mg/kg,	B = 1·5 mg/kg,	C = 3 mg/kg;
(III) embichin,	A = 0·4 mg/kg,	B = 1·5 mg/kg,	C = 2 mg/kg;
(IV) X-rays,	A = 300 r,	B = 600 r,	C = 800 r.

ordinary microscope in white light. The data so obtained were compared with those from fluorescence studies with and without quencher, derived from oil-immersion studies (objective × 90, eyepiece × 10). The cells forming damage foci, and the single cells, were found to be of young granulocyte type (promyelocytes, myelocytes, and metamylocytes), and much more rarely adult forms (with segmented or rod-like nuclei), or lymphocytes. Rarely, and then only at high doses, did we find damaged erythroblasts. If the quencher was omitted, the nuclear and cytoplasmic mucleoproteins were found to be much altered.

As not all cells were damaged equally the structural and fluorescence changes were different; in some cells the nuclei developed coarse structures, with thickened envelopes fluorescing bright yellow, whereas in others the nuclei became homogeneous and contracted, and fluoresced an intense yellow. Cells were often found in which the nuclei presented a ragged contour and had lost their fine-grained structures; these fluoresced dark green. This latter type appeared at later stages (8–9 hr). The break-up of nuclei so often found in spleen, intestine, or transplanted sarcomas in the rat [8] when sarcolysin or dopan are administered, was rare here. Most bone-marrow cells die differently, by lysis and cariotaxis (rare). Hypersegmented leucocytes (7–9 segments) were sometimes found amongst the granulocytes.

Changes also occur in the cytoplasmic nucleoproteins; these appear later than the nuclear changes, or sometimes at the same time. The earliest sign of damage is increased cytoplasmic fluorescence; the red RNA granules vanish. At 7–8 hr the myelocytes and metamyelocytes (rarely lymphocytes) show cytoplasmic spheres of various sizes, which fluoresce dark red. In some cases the spheres fused into one

large mass, which displaced the nucleus to one side. Cells showing naked nuclei (cytoplasm gone) were also seen. The number of cells in these various classes depended on dose and time. The later stages (12–20 hr) at high doses showed very few young granulocytes, and many erythrocytes, which had begun to fluoresce a weak red or brown, were seen; these must therefore also have been damaged.

These changes are similar to those reported by Meisel' and Sondak [1] and by Kondrat'eva [9] for the bone-marrows of X-irradiated animals, or for ones given superlethal (10 mg/kg) doses of embichin [9]. To compare the chemical and radiation effects we did experiments with 300, 600 and 800 r doses of X-rays.

The X-ray results are shown in Fig. 1; the two sets of results do not differ essentially, but at all X-ray doses the numbers of damaged cells were much greater, and damaged cells persisted much longer. Comparable changes were also found in the peripheral blood, in the spleen, and in the lymphatic nodes in both cases.

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DISCUSSION

Our data on the changes induced by chloroethylamines and by X-rays indicate that our method, using acridine orange plus Congo red as quencher, reveals the early changes in the nucleoproteins. Meisel' and Sondak, as also Kondrat'eva, consider these changes, which appear as characteristic cellular conglomerates and individual cells with increased fluorescence when X-rays are used, to be micronecroses. Our data do not enable us to express a final opinion on this, though the changes certainly indicate cell damage, if not death. This is supported by the observation that the damaged cells are mostly young myeloid ones, which soon die and vanish when the chloroethylamines are used. The sizes of the cellular conglomerates are, it is true, somewhat dependent on how the blood is flushed out of the preparation, since if sodium citrate is added to the staining solution the damaged cells appear as separate, or as small groups, with increased fluorescence. The data do indicate that in both cases an important link in the mechanism is early damage to the nucleoproteins.

SUMMARY

(1) Early changes in nucleoprotein metabolism in bone-marrow cells acted on by chloroethylamines are revealed by fluorescence microscopy.

(2) The effects of various chloroethylamines on the bone-marrow function can be compared by this method.

I am indebted to Professor M. N. Meisel' for guidance in this work.

Translated by J. E. S. BRADLEY

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LETTERS TO THE EDITOR

DETERMINATION BY A VISCOSIMETRIC METHOD OF THE MOLECULAR WEIGHT OF DEOXYRIBOSENUCLEOPROTEIN AND OF THE DEOXYRIBOSENUCLEIC ACID CONTAINED IN IT*

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The question of the molecular weight of deoxyribosenucleic acid (DNA) cannot at present be considered solved. The problem is whether the determined molecular weight is characteristic of the natural molecules of DNA, since possibly the molecules we measure have already been partly destroyed, or they have massed together in the process of being extracted.

This question can only be solved by a direct calculation of the molecular weight of DNA which is found in deoxyribosenucleoprotein (DNP) without splitting the latter into its component parts. A number of articles on natural and artificial nucleoproteins have recently shown [1–4] that both in graduated deproteinizing of the natural and in the synthesis of artificial DNP, in spite of the gradual decrease or increase in the quantity of protein, respectively, the characteristic viscosity of nucleoproteins does not change. The constancy of the viscosity of DNP is found over a fairly large range of protein content in DNP (15–25 per cent up to 80–90 per cent) while with a protein content of less than 15–25 per cent a rapid jump occurs from the typical viscosity of DNP to the viscosity of DNA [2, 3]. In our laboratory in a test on artificial and natural nucleoproteins we found [1–3] that the viscosity of DNA was approximately 40–60 per cent greater than the viscosity of DNP, both with graduated deproteinizing of DNP and with syntherizing of the artificial DNA–protein complex. The DNA used was from the liver and the pancreas. A two-fold jump in viscosity was also shown by other authors [5] upon dissociation of DNA from its protein (DNA from the thymus). It is interesting that similar configuration changes of DNA are also observed under the effects of a number of external influences imposed on it [6].

Thus, both from our findings and from facts given in the literature we can conclude that when forming DNP from DNA, at a 15–25 per cent content of protein a point of conversion of DNA to DNP [6] is observed, accompanied by nearly a two-fold reduction of viscosity of the newly formed molecule of DNP in comparison with the original DNA. With further increase in the amount of protein the viscosity of DNP remains constant within fairly wide limits.

How can one explain such changes in configuration accompanied by the two-fold

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change in viscosity? From the formula which expresses the dependence of asymmetry of molecules on their viscosity [7] it is not difficult to see that such changes are brought about by a 30 per cent contraction of the molecule. On the other hand it is known [8] that the transition of DNA from the *B*- to *A*-configuration is also accompanied by a 30 per cent contraction of the molecule. Such configuration changes are thought to occur in the forming of the molecule of DNA and protein. One of us earlier [7] offered the hypothesis that the protein and the nucleic acid in deoxyribosenucleoprotein are in the *a*- and *A*-configurations respectively.

It is true that such changes may also be dependent on purely hydration or configuration changes in the sense of changes of the hydrodynamic form of the molecule by means of reversal of the separate segments. However, the remarkable constancy in the reduction of viscosity and the corresponding contraction of the DNA molecules without regard for the quality of the protein joined to the DNA or for its quantity within rather wide limits (from N/P = 2 to N/P = 15) makes us suppose that the above-mentioned deformation of the molecule of DNA has perfectly definite limits with completely separate end points. We consider that the *A*- and *B*-configurations of DNA are also such separate conditions of DNA.

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As one of us has mentioned [7], for asymmetry of molecules of high polymers (where $a/b \gg 2$) the equation $a/b = 34{\cdot}7\{[\eta]/\bar{v}\}^{\frac{1}{3}}$ can be used with a sufficient degree of accuracy, where $\bar{v}$ is the specific fractional volume. The literature gives the value $\bar{v} = 0{\cdot}65$ cm^3/g for DNP [9] which gives changes of a/b, in comparison with the corresponding asymmetry of DNA of not over 10 per cent. Therefore we can consider that the asymmetry of the molecule of DNP is dependent with a precision within 10 per cent upon the asymmetry of the DNA which forms part of DNP. From this it follows that the two-fold drop in viscosity in the 30 per cent contraction of the DNP molecule (when compared with DNA) is fully dependent upon the configuration changes of the latter when it is united with protein, giving us a basis for using the computing apparatus for DNP which was designed for molecules of DNA.

From all that has been said it is clear that the molecular weight of DNA which forms part of DNP can be expressed by measuring the viscosity of DNP and multiplying the figure by two.

One of us [7] proved that the equation

$$[\eta]_{\text{DNA}} = 9{\cdot}0 \times 10^{-6} M_{\text{DNA}} \tag{1}$$

is satisfied for DNA; from this we get, for DNP,

$$[\eta]_{\text{DNP}} = 4{\cdot}5 \times 10^{-6} M_{\text{DNP}} \tag{2}$$

where $[\eta]_{\text{DNP}}$ is the value of the limiting viscosity of DNP and M_{DNA} is the molecular weight of its component DNA.

Since only one molecule of nucleic acid enters into the composition of one molecule of DNP, we can calculate the molecular weight of this complex by starting with the molecular weight of DNA which enters into it.

Knowing the weight ratio of nitrogen and phosphorous (N/P) of the isolated preparation and taking the percentage of nitrogen in nucleoprotein to be 16 per cent and phosphorous in DNA equal to 9·8 per cent by simple calculation we get as molecular weight of the nucleoprotein $M_{\text{DNP}} = 0{\cdot}607 \dfrac{\text{N}}{\text{P}} M_{\text{DNA}}$ and by putting

the value of the molecular weight of DNA, entering into the composition of nucleoprotein, obtained through the viscosity of DNP, we get:

$$M_{DNP} = 1{\cdot}4 \times 10^5 \, (N/P)[\eta]_{DNA} \tag{3}$$

A comparison of the experimental and theoretical material can be made from the work of Doty and Zubay [5] where the measured molecular weight of DNP by the method of light scattering was $19 \pm 4 \times 10^6$ where $N/P = 3{\cdot}7 \pm 0{\cdot}3$ and $[\eta] = 35 \pm 2$. From equation (3) we see that the molecular weight of DNP can be evaluated at about $18{\cdot}2 \pm 10^6$ only through the given N/P and $[\eta]$. From equation (2) the molecular weight of DNA which forms part of DNP can be evaluated as $7{\cdot}8 \pm 10^6$. This agrees well with the findings of Doty and Zubay who determined this molecular weight by light scattering as $8 \pm 2 \times 10^6$.

We must also consider briefly the writings of Sadron [10] and Pouyet and Weill [11] who mention two types of DNA, called C_1 and C_2, according to the type of function $[\eta] = f(M)$. Most of the known DNA from tissues belongs to group C_1, and follows equation (1). A second and considerably smaller group of molecules of DNA is C_2 and in our opinion is an artifact. These are molecules of DNA which for some reason remained in the tertiary configuration [6] specific for DNP although they are almost totally free of protein. In this case one might expect that the C_2 type of DNA molecules should follow equation (2). In the writings referred to, most of the experimental points on the curves of $[\eta] = f(M)$ follow equation (2).

We therefore conclude from what has been said that the configuration changes of DNP molecules when the latter are formed from DNA and protein, with the resultant doubling of viscosity of DNA over that of DNP, allow us to calculate the molecular weight of DNP ,and of the DNA which forms part of it, without separating the latter from the nucleoproteins.

One can also calculate the asymmetry of DNP using a formula for asymmetry of molecules of DNA [7] (with a 10 per cent margin of error).

We must add that the viscosity of DNP is best measured in 0·5–1 M solution of NaCl, since in 0·2 M NaCl, in which we measure the viscosity of DNA, DNP is precipitated. When measuring the viscosity of DNP, its concentration should not exceed 0·004 per cent. This follows from our previous articles [7, 12].

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CHANGES IN THE PHORESIS NUMBER IN THE PORES OF ISOLATED SKIN OF GUINEA-PIG UNDER VARIOUS PHYSIOLOGICAL CONDITIONS*

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In articles relating to non-living artificial and natural highly porous membranes Zhukov and his colleagues showed that the presence of ions on the surface of the capillaries in equilibrium with the solutions produced the electrokinetic properties of the membranes [1–4]. A charge on the membrane and especially an alteration of of the phoresis number in the pores, in comparison with a free solution, governs the permeability of the membranes to electrolytes [5–7]. The results can be transferred to biological objects since a highly developed system of capillaries containing liquid formed by solutions of electrolytes is found in the bodies of live animals [8–11]. We proved that, having a capacity for ionic exchange, the skin of animals and of man elicits electrokinetic phenomena [6, 12, 13]. The change in the phoresis numbers in the skin is of great importance in the theory of ionophoresis [6, 14] used widely in physiotherapy. Normal skin gives up cations in neutral and alkaline solutions because of the preferential dissociation of acid groups, carries a negative charge, and increases the phoresis number of cations [6].

In clinical practice we have to deal not only with normal but also with altered skin when using ionophoresis. As a consequence of the amphoteric structure of the protein molecule the phoresis number of ions in the skin must depend on the acidity of the solution.

It was of interest to study the phoresis number of ions in the pores of the skin under various physiological conditions with a wide variation of the pH in the solutions including acid solutions.

METHOD

We determined the phoresis number by a method developed by the Department of Colloid Chemistry, Leningrad State University [2, 7]. The phoresis numbers of ions are found from the concentration changes induced by the membrane by direct analysis of the solutions around the membrane. The KCl solution used in our tests had a concentration of 0·01 N (pH 5·8). To obtain a given pH in a solution of KCl, a definite quantity of either HCl or KOH solutions was added to it. The pH of the solutions was measured by a glass electrode. The tests were made on specimens of isolated skin 2 × 2 cm; these were fixed by paraffin wax in a plexiglass ring which was fixed in the flange of the apparatus. Each specimen was used for 2–3 tests. We used skin from the middle part of the back of a guinea-pig epilated directly before

* Biofizika **3:** No. 3, 371–374, 1958 [Reprint Order No. BIO 157].

the experiment. To change the physiological condition of the skin, the latter was treated with agents which cause both inflammation and tanning. The inflammatory phenomena were caused by treating the skin with dinitrochlorobenzene. The sensitizing of the animals with dinitrochlorobenzene was accomplished by the droplet method of Vedrov and Dolgov [15] with the modifications recommended by Pavlov [16]. We used specimens of skin with clear evidence of allergic reactions to the challenging dose of dinitrochlorobenzene.

For tanning we used a 5 per cent solution of $AgNO_3$ and a 40 per cent solution of formaldehyde which were applied daily in 0·03 ml quantities for 7–20 days and 5–12 days respectively.

RESULTS

In the pores of normal isolated skin in a 0·01 N solution of KCl the phoresis number, n_{Cl^-} of Cl^- was found to equal 0·403* as an average of 6 specimens. The variations in individual specimens were $\pm$ 0·006.

In specimens of isolated skin with marked allergic inflammation caused by preliminary application of dinitrochlorobenzene, the n_{Cl^-} was 0·445, taking an average from tests on 7 specimens. When inflammation occurred, the sign of the charge on the skin remained unchanged and the change of the phoresis number was in the direction of an increase of anionic phoresis.

TABLE 1. DEPENDENCE OF n_{Cl^-} ON THE DURATION OF TREATMENT OF SKIN WITH 40% FORMALIN

Days	n_{Cl^-}
5	0·404
7	0·311
10	0·309
11	0·281
12	0·245

With inflammation caused by dinitrochlorobenzene the epidermis became thinner in all its layers. The horny layer was partly torn away and was infiltrated by pseudoeosinophilic leucocytes. The granular layer was partly destroyed. In the basal layer of the dermis were seen foci of leucocytic infiltration, and congestion of the vessels occurred.

Treating the skin with 5% $AgNO_3$ for 7–20 days produced no changes in the phoresis numbers, compared with the value of n_{Cl^-} for normal skin. Skin treated with 5% $AgNO_3$ showed no pathological changes apart from a certain thickening and wrinkling of the horny layer of the epidermis. In Table 1 we give the results of tests on the phoresis number of Cl^- in skin treated with a 40% solution of formalin, according to the number of days the skin was treated with formalin.

Table 1 shows that formalin caused a sudden drop in anionic phoresis in the pores of the skin, which becomes more and more permeable to cations. An analysis of

* In a free 0·01 N KCl solution n_{Cl^-} equals 0·504.

histologic sections of skin treated with formalin showed that fundamental changes from the normal occurred. The horny layer was absent, the cells of the granular and papillary layers were pyknotic, the nuclei stained poorly (due to necrosis and necrobiosis). In the basal layer of the dermis focal infiltrations of pseudoeosinophilic leucocytes arose, especially around the neck of the hair follicles. The vessels were not dilated.

In order to determine the influence of pH of the medium on changes of the phoresis number of ions, tests were made with isolated specimens, both of normal skin and of skin treated with formalin, in a KCl solution with a pH of 2–9. In Fig. 1 we present such findings for two specimens. The curves of n_{Cl^-} vs. pH for normal and tanned skin are parallel. Skin which had been treated with formaldehyde (curve 2) showed a greater change of phoresis number than the normal throughout the pH range in comparison with the free solution. With a value for pH determined at 3·7–2·4 for normal skin and at 4·4–3·2 for skin treated with formalin, the phoresis number of Cl^- did not change. As the concentration of H^+ ions rose in the medium, i.e. when there were shifts toward the acid side from the isoelectric point, the skin became positively charged and increased the phoresis number of Cl^- compared with the free solution.

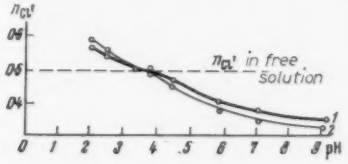

FIG. 1. The dependence of the phoresis number of n_{Cl^-} of Cl^- on the pH of the solution. (1) For normal skin. (2) For skin treated with formalin.

DISCUSSION

The results of tests to determine the phoresis number for Cl^- in the pores of isolated skin confirm the findings of other authors [13, 6] on the negative charge of the skin membrane and the reduction in the anionic phoresis number in the pores when compared with the free solution [6, 14]. When the composition and concentration of the electrolyte were the same the phoresis number in the pores of the isolated membrane was a function of the radius of the capillary [2–4] and as it lessened the phoresis number increased.

The electrokinetic potential was not measured in our tests, but it is hard to believe that the reactions we used changed the composition of the electric double layer. One must assume that this treatment caused a change only in the structure of the capillaries of the skin. When the skin was treated with formalin, a tanning of the proteins and, possibly, a partial constriction of the capillaries occurred. The changes in the phoresis number of Cl^- increased. When dinitrochlorobenzene reacted on the skin congestion was more marked in dilation of the vessels, which evidently led to a lesser change in the phoresis number compared with the normal. The phoresis number of the anion Cl^- in the pores of inflamed skin was larger than in normal skin, approaching the value observed in free solution.

As shown earlier [17] the treatment of gelatinous membranes with a solution of

formalin causes a change in ionic permeability. With different acidities of a solution these changes become qualitative.

In our tests the skin treated with formalin, over the entire range of pH variations, changed its phoresis number in a manner similar to that of normal specimens of skin. The difference was only quantitative; the treated skin changed the phoresis number of Cl^- more than did the normal skin in comparision with a free solution. With a high H^+ concentration in solution, normal and formalin-treated skins behaved as if positively charged; they increased the phoresis number of anions compared with free solutions. An overcharge of skin membrane is imparted by its protein amphoteric nature and, consequently, by dissociation in acid solutions of the basic groups bearing a positive charge.

Explanation of the quantitative differences in the changes of the phoresis number of ions in normal, inflamed, and tanned skin, with capillary structures is based on tests with artificial and natural membranes. In transferring the results and interpretations to live membranes such as skin the peculiarities of the live animal must be allowed for. In our tests the phoresis number was measured on skin isolated from the organism and therefore it would follow the patterns established for non-living or inanimate membranes. However, throughout the test the structure of the skin tissues of the isolated specimens had the same character as in the living animal. Consequently these tests may, to a certain extent, show what phoretic changes occur in the tissues of the animal's body.

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SUMMARY

(1) The change of the phoresis number of Cl^- in isolated skin, when compared with a free solution depended on its physiological condition—whether it was normal, inflamed, or tanned.

(2) The phoresis numbers of Cl^- which were found for normal, and formalin-treated skin changed similarly with variations in the pH of the solution.

Translated by E. L. URCH

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THE DYNAMIC RADIATION INJURY IN *CALANDRA GRANARIA* UNDER VARIOUS IRRADIATION CONDITIONS*

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WE have at present important indicators of the sensitivity of organisms to radiation in the character of increase of the signs of injury, and in the form of the curves of injury. These curves express how many animals died following a given dose of irradiation. One of the main methods of radiobiological research is a study of these functions under various physical and chemical conditions of irradiation. These indicators are fairly well known for many mammals, and for some amphibians, birds and fishes. The question is less clear with insects and until recently literature on the subject expressed doubt about any long-delayed consequences following radiation in adult insects. Nikitin [1] and Sullivan [2] adhered to this view. The latter even mentioned a certain increase in longevity in starved ichneumon flies, *Habrobacon*, which had been irradiated with 180–250 r, in comparison with the non-irradiated controls.

Some authors have mentioned the death of insects some time after irradiation [3–4]. However, up to now, as far as we know, a detailed study of the development of the signs of irradiation injury has not been made.

We have attempted in the present article to determine the regularities of injury when the imagines of insects are irradiated with various doses of ionizing radiation, and to study the dynamic of radiation effects following irradiation under normal conditions and in air with varying amounts of oxygen. To study the effect of ionizing radiation on adult insects we used *Calandra granaria* L. which multiply homodynamically without diapause. γ-rays from radioactive cobalt in a GUT-Co-400 apparatus were used. Death of the insects served as the indicator of radiation injury.

The data we give below are the average from three repeated tests; in all we used 25,000 insects. Each group consisted of 200 adolescent beetles 10–30 days old. The irradiated insects and the non-irradiated controls were kept at 25 °C, with air at 75 per cent relative humidity and with the balanced humidity of the granule at 15 per cent.

The *Calandra* were irradiated in little glass phials with normal aeration at room temperature. Doses used were 0·5, 2, 3, 3·5, 4 and 5 kr with a dose-rate of 230 r/min; and also 25, 75, 100, 150, 179, 225, and 325 kr with a dose-rate of 547 r/min. Count of dead insects was taken every 4 days with doses below 5 kr, and daily with the larger doses.

Curves indicating the deaths in the time of irradiated *Calandra* had an S-shape, and only with doses over 150 kr did they acquire a more nearly exponential form.

* Biofizika **3:** No. 3, 374–376, 1958 [Reprint Order No. BIO 158].

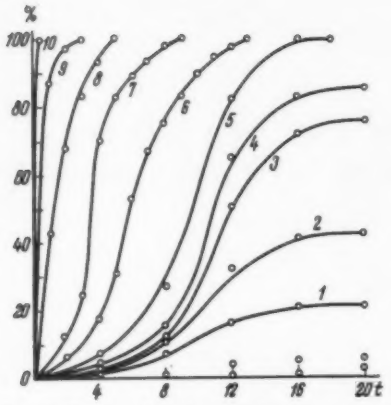

FIG. 1. Curves of deaths of *Calandra granaria* after γ-radiation. Abscissa: the time *t* in days. Ordinate: deaths as a percentage. 1 = 0·5 kr; 2 = 2 kr; 3 = 3·5 kr; 4 = 4 kr; 5 = 5 kr; 6 = 25 kr; 7 = 75 kr; 8 = 150 kr; 9 = 225 kr; 10 = 325 kr.

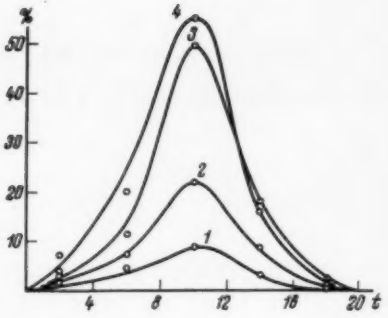

FIG. 2. Distribution curves of the deaths of *Calandra granaria* (in percentage) against the time after irradiation (*t*, in days) 1 = 2 kr; 2 = 3 kr; 3 = 4 kr; 4 = 5 kr.

The 500 r dose caused a negligible number of deaths. The minimum absolutely lethal dose was 5 kr. With 325 kr death occurred during irradiation.

With doses less than 5 kr the distribution curves of deaths in time had one symmetrical peak with a maximum at 10–11 days after irradiation, and, the position of the maximum did not depend on the size of the dose (Fig. 2). When the dose was further increased the points of the crest corresponding to the maximum deaths were displaced to the left along the abscissa in the direction of shorter intervals of time (Fig. 1, curves 6, 7).

A difference in the rate of deaths of irradiated *Calandra* from that of the controls was only seen in the course of the first 20 days after irradiation (with doses of < 5 kr). The survivors did not differ from the controls in their rate of death. Consequently we were able to follow up the dynamic of death only in the first 20 days.

The curve of death of *Calandra granaria* according to the dose had the S-shape typical for the radio-biological effect.

We also found that the radiation sensitivity of *Calandra granaria* depended on the oxygen tension in the air.

For two hours before irradiation and during the whole procedure of irradiation the insects were kept in an atmosphere of oxygen and nitrogen. The oxygen content for the various groups consisted of: 0, 2·5, 5, 10, 21, 75 and 100 per cent. Impurities were removed by bubbling the nitrogen through an alkali solution of pyrogallol and over red hot copper filings. A gasometer, filled with water which was covered with sunflower oil, was used to regulate the oxygen content. The insects were kept in tubes with a capacity of 40 cm^3, and 50 volumes of the gaseous mixture was passed through them. The relative humidity of the gas was brought to 75 per cent by a saturated solution of NaCl. The irradiation dose was 3500 r. The controls were not irradiated. This technique of maintaining insects and the observations on them were the same throughout the later series of tests.

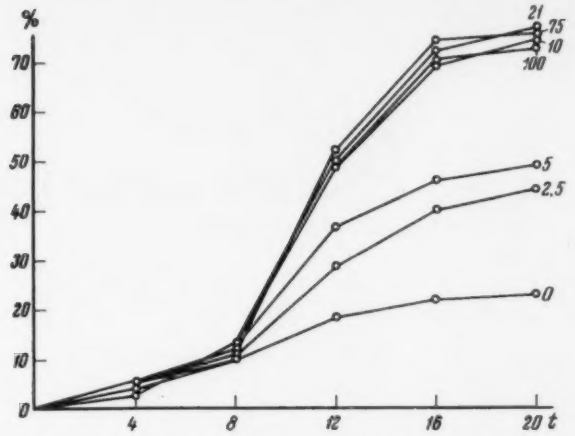

FIG. 3. Curves of deaths of *Calandra granaria* after irradiation (3·5 kr) in an atmosphere with various percentages of oxygen content.
Abscissa: time in days. Ordinate: the deaths as a percentage.

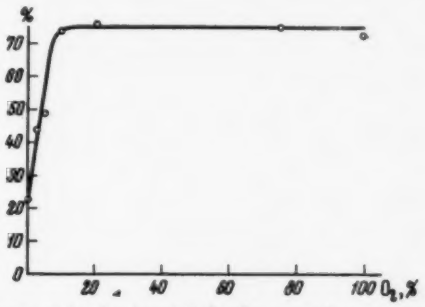

FIG. 4. The dependence of the deaths of *Calandra granaria* over a period of 20 days after irradiation on the percentage of oxygen contained in the medium at the time of irradiation with a dose of 3·9 kr.

The curves of death of *Calandra* (Fig. 3) kept the same S-shape whatever the oxygen concentration during irradiation. After irradiation in an oxygen free medium the deaths were three times less than after irradiation with the same dose in normal atmosphere. An increase of the oxygen content from 0 to 10 per cent caused a greater degree of injury from irradiation among the *Calandra*, but as the concentration of oxygen increased there was no noticeable change in the number of insects irreversibly affected. In Fig. 4 is presented the curve of the deaths of *Calandra* vs. the oxygen content in the medium, during irradiation.* *Translated by* E. L. URCH

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* Thus, these experiments showed that the effects of irradiation on the imago of *Calandra granaria* do not differ basically from the effects on higher animals.

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DISCUSSION

THE NATURE OF THE ELEMENTARY PHOTOREACTIONS OF CHLOROPHYLL*

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A PAPER by Gachkovskii appeared in a recent issue of this journal [1] in which he endeavoured to show that the deductions of Krasnovskii and myself on the reversible photoreduction of chlorophyll (discovered in our laboratory†) were erroneous and contradictory. The first correction to be applied is factual; in Krasnovskii's paper at the conference on chemical kinetics [3], which summarized the work of our laboratory, the main intention was not to justify the "hypothetical"‡ biradical state of chlorophyll, as Gachkovskii supposes. Instead, he presented the results from a systematic study of chlorophyll-sensitized reductions in solutions, in which the chlorophyll was reversibly photoreduced, as first discovered by us.

The essence of Gachkovskii's argument against our interpretation is comprised in two points: (1) that we have not proved that our "hypothetical" biradical state participates in the photoreduction of chlorophyll, (2) that the intermediate product, detected by spectroscopic methods, is not the reduced form of the pigment ("so-called photoreduction"‡), and that the reduction does not, in any case, occur via a conjugated bond system.

The long-lived metastable state into which the pigment molecules can be optically excited is not hypothetical, but has been demonstrated by observation and by comparing the behaviour of the pigment with that of compounds of analogous structure. These observations are (1) that chlorophyll-*b* shows a phosphorescence of maximum emission at 880 mμ [4]; (2) reversible transfer to a metastable state has been observed in the chlorophylls [5–7] and phthalocyanins [8] by spectroscopic methods, intense light flashes being used to produce the excitation. The effect is not found if oxygen (which is paramagnetic) is present, or if the compound contains paramagnetic metal ions; this indicates that the state is of the biradical (triplet) type [8].

Although the biradical origin of the metastable state has only been demonstrated by direct magnetic measurements on other phosphorescent compounds, there is no doubt that chlorophyll is no exception, since nearly all compounds with conjugated bond systems are capable of phosphorescence from a biradical state. Subsequent photo-oxidation and photo-reduction studies using solutions of dyes of very varied

* Biofizika **3:** No. 3, 377–380, 1958 [Reprint Order No. BIO 159].

† This reaction has subsequently been demonstrated by workers abroad, and has come to be called the Rasnovskii reaction [2].

‡ Gachkovskii's expressions.

types [9] confirmed our view that the reacting states of the dyes were not excited (singlet) states, but metastable (biradical*) forms [10]. This approach is now commonly adopted and the concepts have been used to explain the elementary photoreactions of chlorophyll and its analogues [11, 12].

Hence Gachkovskii's assertion that the sole reason for using this approach in this photoreaction is our experiment [13] in which it was found that the reductant has no quenching action on the fluorescence of chlorophyll is unfounded. There is no contradiction between O_2 being able to quench the fluorescence while quinone cannot, while both react. The paramagnetic O_2 facilitates the transition to the biradical state, with which it reacts [14]. Our kinetic studies in this field must give the relative yields of the various elementary reactions quantitatively; the reactions can occur from the excited and from the biradical states of the pigments.

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As regards his doubts on the spectroscopic arguments, to prove that the conjugated bond system of chlorophyll cannot be reduced, we would have to suppose that such a system does not exist in chlorophyll. He has forgotten that there is a difference between the hydrogenation of a semi-isolated double bond (which would give bacteriochlorophyll), which would cause a long-wave shift in the spectrum, and hydrogenation of a conjugated bond system (in particular, at methene bridge points) which leads to leuco-forms and intermediate semi-reduced forms (quinones), which produces a short-wave shift [15]. The conditions of our experiments indicate that we have the latter form, with a maximum at 500–550 mμ, and other maxima, displaced to shorter wavelengths. Abundant data have been given on the spectral evidence for photoreduction in chlorophyll and its analogues [16].

The arguments Gachkovskii gives indicate that he has not understood the essence of the phenomena he is discussing. Some of the references he quotes ([1], p. 757, No. 3) to confirm his argument have no relevance to the topic. Passing to his conceptions as to what occurs, we see that not only are the views and results of Terenin and Krasnovskii erroneous, but so are those of all others who have expressed similar views.† He starts from the idea that the chlorophyll molecule does not exist in an uncomplexed state (as he erroneously asserts all others who have given reaction schemes have assumed) but always exists with molecular addends bound to the Mg. This important circumstance, he considers has not been given adequate attention; he also considers that it indicated the sole correct approach for chlorophyll and its analogues. Unfortunately he gives no concrete reasons why the presence of these addends dispose of our conclusions on the biradical state and conjugated bond system reduction of chlorophyll. He does not even trouble to specify his "important addends" (denoted by A_1, A_2).

That we have merely assigned letter symbols to the reactants does not mean that we have assumed the chlorophyll to have no addends, because chlorophyll, like other pigments, is solvated in solution, and also binds to itself traces of other materials which may be present in an inert solvent. As long ago as 1949 we [17] discovered a highly sensitive spectroscopic test for the binding of water to chlorophyll, bacteriochlorophyll, etc. we demonstrated that the bonding was to the Mg, and

* No other cause of metastability in organic molecules has been demonstrated or is theoretically at all probable.

† Cf. the footnote to p. 758 of [1]; it happens that such "mistaken" workers are very numerous.

rejected an alternative [18] explanation. The main effect of the addends was merely to intensify the existing absorption and fluorescence maxima. The absence of any reference in [1] to these very material results has not escaped our notice.*

Gachkovskii's assertion that these addends on the Mg are highly relevant to explaining the Krasnovskii reaction is immediately disposed of by bringing to bear the experimental results. These pigments, incapable of taking up addends (in the sense of [1], still give the Krasnovskii reaction just the same, quite contrary to Gachkovskii's assertion. Gachkovskii was, of course, well aware of this, as he worked in the same laboratory. The suppression of this important fact shows how reliable his arguments are.

Much of his article is devoted to presenting the experimental basis of his assertion; the experiments described he has repeatedly published previously under various guises [20, 21]. These experiments were done at my suggestion, and were concerned with the effects of various gases and vapours on the *in vacuo* fluorescence spectra of chlorophyll and of Mgphthalocyanin adsorbed on MgO. As the conditions were quite different from those of solution†, and no photoreactions were observed, the results have no bearing on the photoreactions of chlorophyll in solutions containing reductants, and have no direct bearing on the subject of [1]. Their repetition is intended to give the impression that there is some experimental basis for his hypothesis.

These experiments also serve as basis for his highly novel theory of how the spectra of chlorophyll and Mgphthalocyanin arise.‡ In this theory the visible spectra are assigned to the various addends bound to the Mg, such as O_2, H_2O, C_2H_5OH, and $(C_2H_5)_2O$, and not to the conjugated bond system, as is usually assumed; "no addends, no spectra".**

We might seek the logical sources of such a strange supposition in the fact that when molecules co-ordinate with heavy-metal atoms having vacant orbits, the latter are filled, and the corresponding spectra therefore appear. This may hint that spectra can arise by intermolecular electron transfer [22]: chromogenic spectra (produced when certain organic bases combine with porphyrins containing polyvalent cations) are known, as are certain other instances where spectra arise from donor-acceptor interactions. Our attempt to explain rationally how Gachkovskii could have arrived at this theory are, however, fruitless, since metal-free pheophytin and phthalocyanin have structures identical with those of chlorophyll and Mgphthalocyanin, and cannot give addends to the Mg, but still have absorption and fluorescence spectra very similar to those of the Mg pigments.††

That Grachkovskii's theory is inapplicable in the light of current knowledge on molecular spectra does not exhaust our objections. His experimental data on the changes in, and structure of, the fluorescence spectra of chlorophyll and

* This phenomenon has obviously stimulated Gachkovskii to produce his theoretical concepts.

† For example the Mgphthalocyanin adsorbed on MgO was heated to 300°C, while the adsorbed chlorophyll was subjected to prolonged standing with oxygen.

‡ We cannot understand why he does not extend this theory to other dyes and pigments.

** Cf. for instance [21]. In [19] a figure showing an O_2–Mgphthalocyanin complex is given, the O_2 being represented as $-0^+=0^+$ and as being bound covalently to the Mg, the bond energy being given as 85 kcal/mole (!). It is difficult to take such absolutely unfounded hypotheses seriously.

†† Quantum-mechanical calculations on the spectral structures for the "bare" porphyrin skeletons (no central metal atoms or addends) agree well with experiment [23].

phthalocyanin adsorbed on MgO, which are given in [1], and which I have in my time forwarded for publication in other papers [22], were so difficult for him to produce that the experiments had to be repeated by colleagues in other laboratories. These experiments, when repeated and extended using quantitative photoelectric spectrophotometry [24] instead of photographic methods, gave results which were different from those described; they also allow of a more simple and natural interpretation than Gachkovskii puts on them [1, 19, 21].*

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SUMMARY

The idea (advanced in a discussion in this journal [1], that oxygen-containing addends to the Mg atom are exceptionally important in the reversible photoreduction is disproved by the fact that the reaction goes just as well with pigments having no central metal atom. The spectral curves and the arguments against the possibility of reduction in the conjugated bond systems in the pigments, which are given in [1] are unsound. The arguments against the photo-active form being a biradical are unacceptable; his assertion that the biradical state is "hypothetical" and that there is only one piece of positive experimental evidence supporting the "assumption", are disproved by quoting the numerous data which point to a biradical form. The presence of addends on the Mg does not prevent the biradical state arising in chlorophyll, and does nothing to explain the photoreaction mechanism, since Mg-free chlorophyll (pheophytin) reacts just as well. Gachkovskii's theory [1, 19, 21, etc.] of the origin of the spectra of chlorophyll and Mgphthalocyanin (the result of addends such as O_2, H_2O, C_2H_5OH and $(C_2H_5)_2O$ being bound to the central Mg atom) is without basis in logic or experiment.

Translated by J. E. S. BRADLEY

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* The smooth spectral curves of [1, 20, 21] were traced manually from the photographs, and are not microphotometer curves for the blackening referred to identical exposures.

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ON NIUBERG'S ARTICLE "COLORIMETRIC EXPERIMENTS AS A MEANS OF STUDYING COLOUR VISION: THEIR BASIC REQUIREMENTS"*†

N. G. BOLDYREV, M. M. GUREVICH, P. M. TIKHODEEV, and N. T. FEDOROV

THE article by Niuberg [1] touches on the basic tenets of colorimetry (more exactly called the lower metrics of colour) and could be of interest in and of itself. In view of recent publications on efforts to revise the curves of the composition of colours for the average eye and to study their connections with the diameter of the field of vision, a number of situations may develop which are seldom examined in print and are not always fully clear. If the author had been able to give new facts impartially and objectively, the article could have been a useful one. Unfortunately we have to conclude that in this case the author failed in his approach. Possibly this is because Niuberg presented his findings polemically, concluding his article with a characteristic remark: "If this had not been overlooked so frequently our discussion would not have been required".

This polemical style could be the reason for the unacceptable carelessness in

* Biofizika **3:** No. 3, 381–383, 1958 [Reprint Order No. BIO 160].

† This was written immediately after publication of the second number of *Biofizika* in 1957, containing N. D. Niuberg's article, but the editorial board held back its publication because the conference of the working committee of the International Illumination Committee (I.I.C.) on colorimetry (1–3–1) was to take place in London in September of that year. We can now state that the resolutions made in London only confirmed the accuracy of what has been written. The working committee of the I.I.C. on colorimetry (1–3–1) showed that (1) in tests known at present, with a metametric measurement at the polar field less than 4°, no significant differences have been found from the standards of observation predicted in 1931; and (2) efforts must be made to maintain a relationship between the colorimetric and the photometric measurements and to put the function $V(\lambda)$ before the Committee (1–4–1) for the 10° field which satisfies this requirement.

It was also decided that, for international comparison, the colour co-ordinates of luminescent lamps must be calculated in the XYZ co-ordinate system according to the measurements of the light spectrum based on the curves of composition of the standard observer of the I.I.C. in 1931.

presenting the works of other people to the extent of directly distorting them. Thus Niuberg writes that Wright defined the curves of composition for various observers in such a way that for all of them the "white" rays would always be described by the numbers 1 : 1 : 1, and that "this is obtained by all the readings of each observer being divided by the readings which *this* observer [italics ours] has made when balancing mixtures of the three basic colours with white". This is precisely what Wright did not do [2].

Similar mistakes are made earlier in the article, such as his unfounded criticism of the method of constructing curves of trichromatic coefficients, which Wright uses and which is an extremely accurate method [3].

What are these forgotten truths which Niuberg seeks to re-establish and with whom is he conducting a discussion? Let us look at the article. In concluding its first paragraph the author writes: "There is no proper clarity in the literature on the subject about what exactly should be recorded and measured when colorimetric experiments are performed. This is the source of many sad misunderstandings. One of the most striking examples of this is the International Colorimetric Standard of 1931. At the conference in Zurich in the summer of 1955 [4] it was finally acknowledged officially that the *experimental findings* [italics ours] used as a basis of the standard had been obtained by an imperfect method, since Guild and Wright, who investigated them, measured the wrong entities. This is by no means the only example".

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This quotation shows that the author reports the meaning of conclusions of the I.I.C. in 1955 totally incorrectly. Experimental facts used as a basis of the standard of the I.I.C. in 1931 had been obtained by an infallible method, the apparatus producing identical visual results. He should also know that in 1955 Stiles repeated Guild and Wright's experiments with totally different apparatus, and by a different method with exact energy measurements obtained for the trichromatic coefficient for *all* wave lengths over 488 mμ results "hardly distinguishable from the curves of the I.I.C." For wave lengths less than 488 mμ the differences were slightly (but insignificantly) greater; this could be explained by the fact that with Stiles, the 2° test field lay on a background of an angle of 14° of the same colour and brightness.

This is the exact meaning of the I.I.C. recommendations concerning this matter:

(1) to draw the attention of national committees to study the curves of blending in a field of vision of 10°, and

(2) to recommend that new curves of blending be determined on the basis of complete colorimetric equality established by the observer in the field of vision [6].

This is a far cry from discounting the work of Guild and Wright as inadequate.

Even when we consider that as Professor Richter writes [7] the real meaning of these resolutions consists in the fact that when one goes over from trichromatic coefficients to curves of blending one should not use the curve of the spectral sensitivity of the eye, this still gives no basis for affirming that the I.I.C. officially acknowledge Guild and Wright's work to be imperfect. The same author [7] writes a little further on that " both research workers without any doubt measured and calculated with meticulous care".

Niuberg persists in repeating his own version that the work of Guild and Wright is at last acknowledged as incorrect. For instance on p. 160 he writes "The conference

of the International Commission on Illumination in Zurich acknowledged in 1955 the *unsatisfactoriness* [italics ours] of the standard based on Guild and Wright's findings and we welcome this warmly".

The author sees the "imperfection" of Guild and Wright's determination of curves of composition (p. 157) in the fact that both of them used "subjective comparisons only with respect to brightness" of illuminations which were visually different, and recalculations according to the so-called "curve of visibility".

The author calls this method "imperfect" because "an essential violation of additivity for heterochromic comparisons was revealed".

However no deviations from the additivity of brightness can diminish the value of Guild and Wright's determinations of trichromatic coefficients for spectral colours. As for the additivity of spectral brightnesses, i.e. the question whether a curve of visibility can be obtained as the linear combination of curves of composition, irrespective of one or the other solution to this question, the following opinion of Judd, president of the committee on colorimetry of the I.I.C. is interesting. He writes in a commentary to the resolutions of the Zurich Session that the I.I.C. had decided to examine the curve of visibility (of light effectiveness) as a certain function of wave length acceptable rather for its convenience and usefulness than because the brightness expressed by this curve should exactly correspond to the one actually seen by the eye. He also writes that the acceptance of any weighted sum of functions of the composition of colours as the function of colour effectiveness would correct the undesirable situation created by experimentally observable deviations from the rule of the additivity of brightnesses [4].

One should remember that Wright himself in his book "Photometry and the Eye" [8] published in 1949 wrote "There is too great a tendency to consider that the old, and to a certain extent non-critical, experiments confirmed additiveness more exactly than was really the case, resulting in additiveness being almost universally taken as an axiom".

In the next to last paragraph in his article, Niuberg confirms that "much harm was done to colorimetry by the obstinate striving to separate the 'brightness' characteristic from 'colouring' ". In actual fact, from the time of Newton and Grassman up to our contemporary Schrödinger the quantitative characteristic of a colour has always been separated from the qualitative. Such a separation is possible precisely because of the basic laws of colorimetry, for instance the position put forward by Niuberg on p. 156 with the designation of one of the results of Grassman's rule of additiveness.

As a quantitative measure of colour one takes not the energy values but the sum of the integrals:

$$\int e(\lambda)a_1(\lambda)\mathrm{d}\lambda + \int e(\lambda)a_2(\lambda)\mathrm{d}\lambda + \int e(\lambda)a_3(\lambda)\mathrm{d}\lambda$$

where $a_1(\lambda)$, $a_2(\lambda)$ and $a_3(\lambda)$ are ordinates of the curve of composition and $e(\lambda)$ is the ordinate of the curve of spectral distribution of energy; it is not essential for this curve to be expressed in any absolute energy units. Therefore if a certain weighted sum of the functions of composition be introduced as a function of light effectiveness, as Judd suggests, there can be no doubt about the possibility and practical desirability of separating the "brightness" characteristic from the "colour".

On p. 160 Niuberg makes some exceedingly strange remarks to the effect that the determination of the trichromatic coefficients "deprives the results obtained of any theoretical and practical meaning". Everybody knows that the trichromatic coefficients are the essential part both of the lower metrics of colour generally and of a table accepted internationally which characterizes the properties of the normal observer. The severe judgment mentioned above only introduces confusion into the ideas of the less qualified reader.

The trichromatic coefficients are used in practice to solve a number of colorimetry problems, such as, for instance, Kustarev's [9] most interesting articles on the graphic conversion of the co-ordinates of hue and of colour printed in (Svetotekhnika) in 1956 and 1957. It is also worth remembering that Schrödinger, whom Niuberg evidently considers capable of understanding the elementary basis of colour measurement in his review article on the work of Guild on the geometric solution of the problems of colour blending [10], found nothing incorrect in his using trichromatic coefficients in colorimetry.

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On p. 158, Niuberg, when speaking of the influence which individual cases of colorimetric conditions of comparison may have on the curves of blending, considers it possible to claim that observed divergences of the observations made "are, in any case, not large". But his claim is wrong.

If the field of vision in which the colorimetric comparison is being made is sufficiently small (10–30) then we know [11, 12] that the curves of blending undergo a substantial change because within the limits of small fields each normal observer is a tritanope.

Confusion in formulation becomes obvious. There is a sentence in the conclusions: "Only colorimetric experiments on the complete indistinguishability of fields answer in an exhaustive way the question of which illuminations are visually distinguishable". From this it follows that instead of looking at the sky and deciding that the white clouds are "visually distinguishable" from the blue sky one must use a colorimetric experiment on the indistinguishability of fields and only such an experiment.

We must conclude by saying that although Niuberg presents some correct statements, which, however, by themselves are not in any way new in principle, on the whole his article gives an obviously incorrect presentation to non-specialist readers of the true state of affairs in the field of colorimetry and causes justifiable amazement in specialists.

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THE BRAIN AS A SYSTEM OF AUTONOMIC "MEMORY"*

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In this paper the hypothesis is put forward that from the physico-mathematical point of view the brain is a collection of non-linear autovibratory systems with feedback connexions, described by functional equations with a retarding argument. The action of the systems is electrochemical. The problem is considered of one of the main functions of the brain—human memory. The mathematical theory of a dynamic "memory" is given. Analysis shows the existence of two systems of autonomic "memory": (1) a system of absolute "memory", (2) a system of relative "memory". The hypothesis is put forward that the brain possesses a dynamic relative "memory".

Neuman, in his paper "Probability logic and the synthesis of reliable organisms from unreliable components" [1], considers the question of the probability of different hypotheses concerning the numerical or modelling character of the nervous system. At the beginning of the article the question is put of the so-called frequent automata (or frequent schemes). Further, Neuman poses the question: are not these frequent schemes (or something very like them) a characteristic feature of the actual human nervous system (or more generally, of the animal nervous system)? Neuman replies to this question that no categorical answer can be given. He sees the main difficulty in the fact that such systems blindly follow the method of construction which is dictated by traditional concepts of mathematics and mathematical logic. A logical scheme for some of the important features of the nervous system differs qualitatively and quantitively from our usual concepts in mathematics and mathematical logic. Neuman does not deny the existence of feedback connexions in the nervous system; the nerves are bundles of nerve fibres and the nervous system contains numerous collections of nerve cells.

These considerations led us to seek a more profound analogy, not of a purely mathematical but of a physico-mathematical character. We tend to the view that it would be of great interest to clarify this problem by the analysis of one of the fundamental functions of the brain—the function of memory. We consider that the brain is a collection of non-linear auto-vibratory systems with a reverse communication. We will deal briefly with the block diagram of these systems and then consider the question of "memorizing" an impulse of arbitrary form. Such systems are systems of dynamic "memory", or systems of autonomic "memory". So far the theory of "memory" of the above-mentioned systems has not been described in the literature, and there is only one report dealing with the problem [2].

Autovibratory systems must have a feedback chain for the transmission of energy

* Biofizika **3**: No. 4, 385–390, 1958.

back into the system. Two groups of systems are distinguished, corresponding to different block diagrams. The block diagram of the first group (Fig. 1) consists of a source of constant energy, a non-linear amplifier of signals I, a vibratory system II and a chain of delayed feedback III. The block diagram of the second group (Fig. 2) consists of a source of constant energy, a non-linear amplifier of signals I, and a feedback chain II. In Figs. 1 and 2 are represented block diagrams for changing the component vibrations. We may note that actual feedback chains possess dispersive properties, i.e. cause a certain amount of distortion. Nevertheless in a number of cases the chains may be considered ideal. We will examine non-linear autovibratory systems, whose motion can be described by functional equations with a retarding

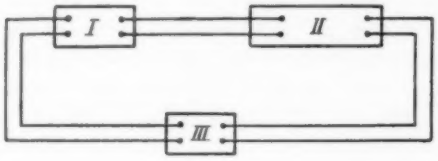

FIG. 1.

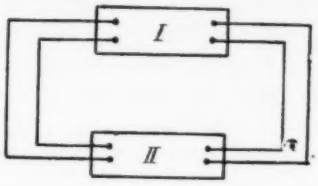

FIG. 2.

argument of the form*

$$x(t) = A[x(t)]x(t-\tau), \tag{1}$$

where $A[x(t)]$ is a given continuous function of $x(t)$ in which $t \geq 0$, and $\tau > 0$ is of constant magnitude ("retarding" systems). In the future we will confine ourselves to regard the systems with A in the form of a polynomial:

$$A[x(t)] = a_0 + a_1 x(t) + a_2 x^2(t) + \ldots + a_n x^n(t), \tag{2}$$

where $a_0, a_1, a_2 \ldots a_n$ are constants and $n \geq 1$.

It might be supposed that collections of nerve cells in the brain correspond to the component parts of the above block diagrams, that the nerves are the conductors, and that the action of the system is electrochemical. We will now examine the question of the intrinsic vibrations of such a system in the form of stepped impulses. We may assume that the periodic solution (motion) of the functional equation (1) (system) is

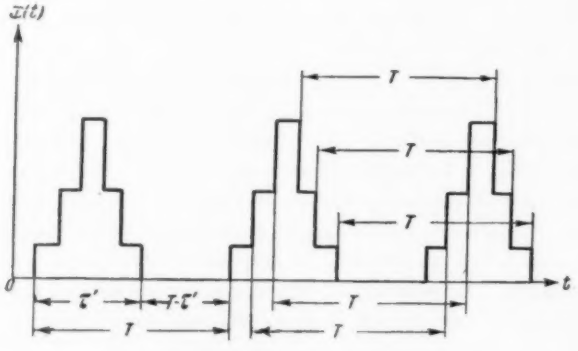

FIG. 3.

*All the results will also conform to the systems: $x(t) = A[x(t-\tau]x(t-\tau)$.

a periodic sequence of stepped impulses of arbitrary duration τ' and with a period T (Fig. 3). Not every impulse must possess an axis of symmetry (Fig. 4). Let the values of the heights of the steps of the impulse be $A_1, A_2 \ldots A_k$ $(k \geqslant 1)$. We shall show that in any conditions such a sequence of impulses can be realized if

$$T = \tau. \tag{3}$$

It may be shown that in the system (1) (functional equation) there exist periodic movements (solutions) having a necessary period T, divisible by the retardation of the system τ. Since the stepped impulse consists of a collection of rectangular impulses it will be sufficient to prove this for the case of a rectangular impulse, in particular by the method of consideration of the contrary.

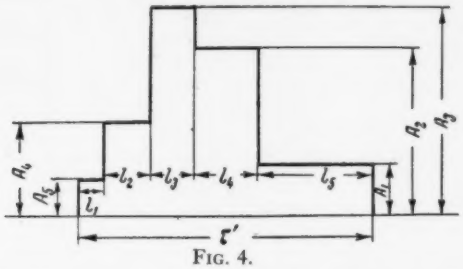

FIG. 4.

When $\tau > \tau'$ it is clear that in this case the following must be true:

$$A_1 = (a_0 + a_1A_1 + a_2A_1^2 + \ldots + a_nA_1^n)A_1 \tag{4}$$

$$A_2 = (a_0 + a_1A_2 + a_2A_2^2 + \ldots + a_nA_2^n)A_2 \tag{5}$$

$$A_k = (a_0 + a_1A_k + a_2A_k^2 + \ldots + a_nA_k^n)A_k \tag{6}$$

or thus:

$$a_nA_i^n + a_{n-1}A_i^{n-1} + \ldots + a_1A_i + a_0 - 1 = 0 \tag{7}$$

$$(i = 1, 2, 3 \ldots k).$$

Consequently the value of A_i must be the actual positive solutions of equation (7). If the values of $A_i (i=1, 2, 3 \ldots k)$ are given, it will always be possible to select suitable parameters of the system $a_j (j=0, 1, 2 \ldots n)$ and the index n. In particular we can make $n=k$ and make use of the Vieta formula (see [3]):

$$\frac{a_{n-1}}{a_n} = -(A_1 + A_2 + \ldots + A_n) \tag{8}$$

$$\frac{a_{n-2}}{a_n} = A_1A_2 + A_1A_3 + \ldots + A_1A_n + A_2A_3 + \ldots + A_{n-1}A_n \tag{9}$$

$$\frac{a_{n-3}}{a_n} = -(A_1A_2A_3 + A_1A_2A_4 + \ldots + A_{n-2}A_{n-1}A_n) \tag{10}$$

$$\frac{a_1}{a_n} = (-1)^{n-1}(A_1A_2 \ldots A_{n-1} + A_1A_2 \ldots A_{n-2}A_n + \ldots + A_2A_3 \ldots A_n), \tag{11}$$

$$\frac{a_0 - 1}{a_n} = (-1)^nA_1A_2 \ldots A_n. \tag{12}$$

Consequently one of the coefficients $a_j (j=0, 1, 2 \ldots n)$ may be selected arbitrarily and the remaining coefficients determined singly from the formulae (8)–(12). For periodic motion in the system it is necessary that the function of the initial impulse

$x(t)=\varphi(t)$ should periodically prolong the motion of the system for the initial length of time τ (Fig. 5), i.e. in this case at the initial value of time $E_{t_0}(t_0-\tau \leqslant t \leqslant t_0)$ there must be only one impulse. In a particular case, if this impulse is a rectangle ($k=1$), the initial value of E_{t_0} conforms to one rectangle (Fig. 6), while in the case represented by $t=t_0$ the next impulse must begin.

It is always possible to draw up approximately the functional equation of a non-linear vibratory system with retardation, whose intrinsic vibrations must represent a periodic succession of impulses of arbitrary form and arbitrary duration τ' with a

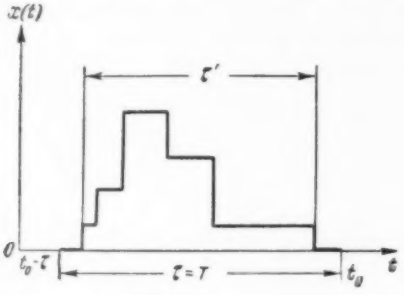

FIG. 5.

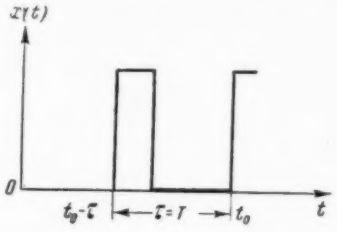

FIG. 6.

perfectly definite period T (Fig. 7). For this purpose it is sufficiently approximate to replace the given impulse by a stepped one (Fig. 8), to determine $A_i(i=1, 2, 3 \ldots n)$ and the ratios $a_i/a_n(i=1, 2, 3 \ldots n-1)$ and $(a_0-1)/a_n$. The retarding systems must be selected with an equal retarding period T, i.e. $\tau=T$.

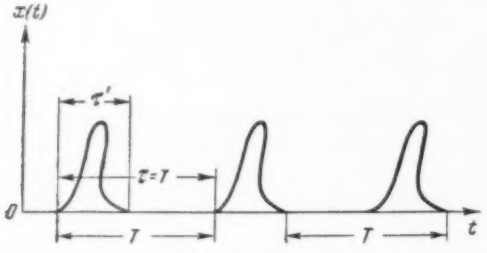

FIG. 7.

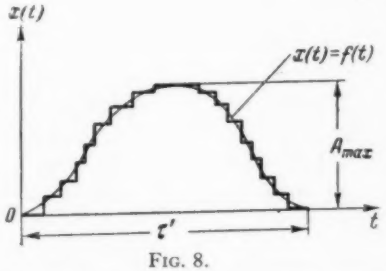

FIG. 8.

Let us now move on to the limit. As $n \to \infty$, the equation of the vibratory system with periodic motion of an impulse of arbitrary form and with a retarding period T will be:

$$x(t) = \mathrm{F}[x(t)]x(t-\tau), \tag{13}$$

where $\mathrm{F}[x(t)]$ is some continuous function of $x(t)$, in the form of a graduated descending series $\mathrm{F}[x(t)]=\sum_{i=0}^{\infty} a_i x^i(t)$ for values $0 \leqslant x(t) \leqslant A_{\max}$ where $A_{\max}$ is the height of the impulse (Fig. 8). We may note that in place of equation (7) we have*:

$$\mathrm{F}[x(t)]-1 = 0, \tag{14}$$

* This comes about as follows: the values of the function $\mathrm{F}[x(t)]$ at all points of the interval $0 \leqslant x(t) \leqslant A_{\max}$ (at any $A_{\max}$) must be constant and equal to unity. Consequently in the region of determination of the function F, $\mathrm{F}[x(t)]=1$ (for any value of $A_{\max}$) and the equation of the vibratory system will be $x(t)=x(t-\tau)$.

i.e. the equation of the vibratory system is:

$$x(t) = x(t-\tau). \tag{15}$$

Such a system is called an identical vibratory system (a system of absolute "memory"). To return to the stepped impulse. If the given equation (1) and the algebraic equation (7) possess certain material positive roots, k in number ($k \leqslant n$), then in the non-linear system there must pass a periodic succession of stepped impulses with a period T, equal to the retardation of the system; $T=\tau$; and moreover the duration of the impulse τ' is restricted by the inequality:

$$\tau' \leqslant T. \tag{16}$$

However, the lengths of the individual areas, i.e. the durations $l_j (j=1, 2, 3 \ldots k)$ of discrete values of the impulse $A_j (j=1, 2, 3 \ldots k)$, may be of any value so long as they satisfy the equation:

$$\sum_{j=1}^{k} l_j = \tau'. \tag{17}$$

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Consequently the unending multiplicity of forms of the impulse with different values of τ' and different durations l_j of discrete values of the impulse corresponds to the given functional equation (1) with a retarding function F in the form of a polynomial. The function $F[x(t)]$ in the case of a polynomial determines the height A_j of the steps of the impulse. In the case of a stepped series its sum is equal to unity, i.e. $F[x(t)]=1$, and the system "memorizes" impulses of any form even without restriction of its maximum height. In this case a periodic succession of impulses of arbitrary form in

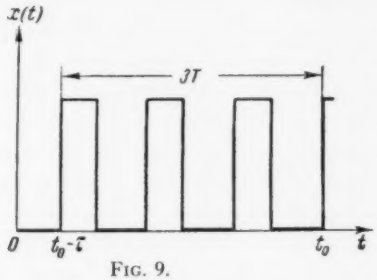

FIG. 9.

a non-linear system (1) which, it is true, degenerates essentially into a linear system $x(t)=x(t-\tau)$, may be realized by the same form of function of the initial impulse. This is evidence of the important role of "neglect" of the intrinsic vibrations of the systems under examination. So far we have considered the case where the retardation τ is equal to the period of vibration T. It is not difficult to ascertain that all the previous results are entirely true in the case in which the retardation of the system τ is an integral multiple of T:

$$\tau = rT \, (r=1, 2, 3 \ldots). \tag{18}$$

In this case at the initial length of time τ it is essential to produce r impulses of the given form. In Fig. 9 is shown the case of a rectangular impulse where $r=3$.

These non-linear vibratory systems belong to the group of autovibratory systems, and are systems of dynamic storage of information in the form of signals. These

systems are also conveniently called systems of autonomic "memory" or of dynamic "memory". On the basis of the examination we have made, we may conclude that if we created a non-linear vibratory system (1), "memorizing" some given impulse of determined form with a maximum height A_{max}, and accurately replacing it with a stepped impulse (in the sense of levels), then this system would "memorize" with the same accuracy impulses of any other form with a maximum height A'_{max}, with the condition that $A'_{max} \leqslant A_{max}$.

Identical vibratory systems are thus systems of absolute memory (for "memorizing" an uninterrupted process). All the true non-linear systems of autonomous "memory" are systems of relative "memory", i.e. they "memorize" a continuous process in the form of a succession of discrete points (corresponding to the level of the impulse). Systems of absolute "memory" may "memorize" any impulses of arbitrary form without restriction of their amplitude. We consider that the brain consists of a collection of a non-linear vibratory systems of relative "memory" and the more the system of relative "memory" tends towards a system of absolute "memory", the better the memory of the brain (in the sense of detail) (i.e. as n increases, the moduli of the parameters of non-linearity $|a_2|$, $|a_3|$, . . . $|a_n|$ diminish, tending to a limit of zero as $n \to \infty$, and also the modulus of the parameter of linearity $|a_1|$ ($|a_1| \to 0$.) Thus the brain possesses a dynamic memory. The capacity of memory of the brain is greater, the greater the collection of vibratory systems mentioned above. Selection of the required impulses (i.e. recollection) takes place by means of a command which the person transmits to the appropriate area of the brain (in the transmission of this command an important part is evidently played by the tongue, the means of creating the sounds of speech at will). The impulse reaches the outer chain and further, to the tongue, and the recollection takes place audibly or mentally, and may be reproduced simultaneously in writing. It must be mentioned further that during the passage of the impulse through the corresponding brain cells, carrying out the function of the vibratory systems, non-linear amplifiers, and feedback chains, certain changes take place in the structures of these cells; the changes remain unaltered in time. Although, therefore, the impulse is preserved by way of reproduction, the changes in structure of the cells are static. If for various reasons the feeding of these systems with current is suspended for a short period, then on its restoration there is recovery in the vibratory systems of those impulses which correspond to the residual static changes in the cell structures. Maintenance of the function of memory by means of repetition pursues two objects: (1) in view of the unavoidable losses during movement of the impulse in the vibratory system from time to time it becomes weak (and sometimes completely disappears), and it is necessary to reproduce it; (2) passage of the impulses is associated with static changes in the structure of the cells, therefore maintenance of memory is effected by means of repetition in order to obtain a highly static condition of the changes in cell structure. Consequently although in our view the memory of the human brain is dynamic, it is associated with perfectly defined static changes in the structure of the brain cells, and so taken all in all it bears a dynamico-static character. However, the conception of the memory of the brain which has been put forward does not deny the possibility of another, purely static memory in isolated areas of the brain.

Translated by B. HAIGH.

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THE PRIMARY "ALPHABET" OF DEOXYRIBOSENUCLEIC ACID*

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IT is known that after the discovery of the comparatively simple structure of deoxyribosenucleic acid (DNA) [1–4] the question arose of the deciphering of the "code" by means of which DNA, in material form, has the power to attract or become associated with certain amino acids to form proteins. The hypothesis put forward in certain papers [5, 6] did not solve the problem.

We suggest a concrete scheme to explain the way in which 3 bases inside the cell jointly determine one of the 20 letters of the "alphabet" of DNA. We suggest that the DNA cell consists of 3 bases in a line along the DNA. The combined cell carries the same information as do its components (see below). Since in each section of the cell one of the 4 bases A, G, T and C may be found, evidently there may be altogether 64 different combinations of making up the full complement of bases in the cell. Thus we could have 16 combinations for cells having adenine in the first section:

$$\begin{Bmatrix}A\\A\\A\end{Bmatrix}\quad\begin{Bmatrix}A\\A\\G\end{Bmatrix}\quad\begin{Bmatrix}A\\A\\T\end{Bmatrix}\quad\begin{Bmatrix}A\\A\\C\end{Bmatrix}$$

$$\begin{Bmatrix}A\\G\\A\end{Bmatrix}\quad\begin{Bmatrix}A\\G\\G\end{Bmatrix}\quad\begin{Bmatrix}A\\G\\T\end{Bmatrix}\quad\begin{Bmatrix}A\\G\\C\end{Bmatrix}$$

$$\begin{Bmatrix}A\\T\\A\end{Bmatrix}\quad\begin{Bmatrix}A\\T\\G\end{Bmatrix}\quad\begin{Bmatrix}A\\T\\T\end{Bmatrix}\quad\begin{Bmatrix}A\\T\\C\end{Bmatrix}$$

$$\begin{Bmatrix}A\\C\\A\end{Bmatrix}\quad\begin{Bmatrix}A\\C\\G\end{Bmatrix}\quad\begin{Bmatrix}A\\C\\T\end{Bmatrix}\quad\begin{Bmatrix}A\\C\\C\end{Bmatrix}$$

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For cells having in the first section G, T and C, we obtain a further 48 combinations. However, it is known that there are 20 amino acids, and consequently it was necessary for us to show that for construction of the amino acids only 20 combinations exist out of 64.

We may use two postulates:

I. Cell α and cell α^* combined with it carry identical "information" loads and consequently α and α^* are in association with the same amino acid. The cell α^* is combined with α if the elements contained in α^* were obtained by means of replacement in α of A by T, T by A, G by C and C by G.

II. Cell α and cell α^{-1}, which contain bases which are mirror images in relation to the middle section of the cell α, carry identical "information" loads and consequently α and α^{-1} are associated with the same amino acid.

If $\alpha^{-1} \neq \alpha$, then $(\alpha^{-1})^* \neq \alpha^*$; consequently only two cells α and α^* bear the same genetic load.

If $\alpha^{-1} = \alpha$, then $(\alpha^{-1})^* = \alpha^*$; consequently four cells α, α^*, α^{-1} and $(\alpha^{-1})^*$ bear the same "information" (genetic) load.

Let us consider an example where $\alpha = \alpha^{-1}$. To these elements, for example, belong:

$$\begin{Bmatrix} A \\ A \\ A \end{Bmatrix} \quad \begin{Bmatrix} G \\ G \\ G \end{Bmatrix} \quad \begin{Bmatrix} T \\ T \\ T \end{Bmatrix} \quad \begin{Bmatrix} C \\ C \\ C \end{Bmatrix}$$

$$\begin{Bmatrix} A \\ G \\ A \end{Bmatrix} \quad \begin{Bmatrix} A \\ T \\ A \end{Bmatrix} \quad \begin{Bmatrix} A \\ C \\ A \end{Bmatrix} \quad \begin{Bmatrix} G \\ A \\ G \end{Bmatrix}$$

$$\begin{Bmatrix} G \\ T \\ G \end{Bmatrix} \quad \begin{Bmatrix} G \\ C \\ G \end{Bmatrix} \quad \begin{Bmatrix} T \\ A \\ T \end{Bmatrix} \quad \begin{Bmatrix} T \\ G \\ T \end{Bmatrix}$$

$$\begin{Bmatrix} T \\ C \\ T \end{Bmatrix} \quad \begin{Bmatrix} C \\ A \\ C \end{Bmatrix} \quad \begin{Bmatrix} C \\ G \\ C \end{Bmatrix} \quad \begin{Bmatrix} C \\ T \\ C \end{Bmatrix}.$$

It is obvious that any of these 16 cells is invariant in relation to the operation of mirror reflection, i.e. the transfer of a base from the third section to the first and from the first to the third.

On the other hand, in accordance with postulate I the cell $\begin{Bmatrix} A \\ A \\ A \end{Bmatrix}$ bears the same characteristics as cell $\begin{Bmatrix} T \\ T \\ T \end{Bmatrix}$ since the base T is combined with A. This combination may for brevity be signified * (similar to the symbol of complex combination in mathematics).

We have

$$(T)^* = A,\ (C)^* = G,\ (A)^* = T,\ (G)^* = C.$$

On the basis of postulate I there are 8 pairs of combinations of types α and α^* with identical "information" load.

First pair $\begin{Bmatrix}A\\A\\A\end{Bmatrix}$ $\begin{Bmatrix}T\\T\\T\end{Bmatrix}$ Second pair $\begin{Bmatrix}G\\G\\G\end{Bmatrix}$ $\begin{Bmatrix}C\\C\\C\end{Bmatrix}$

Third pair $\begin{Bmatrix}A\\G\\A\end{Bmatrix}$ $\begin{Bmatrix}T\\C\\T\end{Bmatrix}$ Fourth pair $\begin{Bmatrix}A\\T\\A\end{Bmatrix}$ $\begin{Bmatrix}T\\A\\T\end{Bmatrix}$

Fifth pair $\begin{Bmatrix}A\\C\\A\end{Bmatrix}$ $\begin{Bmatrix}T\\G\\T\end{Bmatrix}$ Sixth pair $\begin{Bmatrix}G\\A\\G\end{Bmatrix}$ $\begin{Bmatrix}C\\T\\C\end{Bmatrix}$

Seventh pair $\begin{Bmatrix}G\\T\\G\end{Bmatrix}$ $\begin{Bmatrix}C\\A\\C\end{Bmatrix}$ Eighth pair $\begin{Bmatrix}G\\C\\G\end{Bmatrix}$ $\begin{Bmatrix}C\\G\\C\end{Bmatrix}$

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We might be able, between complexes of the pairs enumerated, to set up a sign of mathematical identity, considering that it is in fact only in relation to genetic load (attraction of the same amino acid).

And so,

$$\begin{Bmatrix}A\\A\\A\end{Bmatrix} \equiv \begin{Bmatrix}A\\A\\A\end{Bmatrix}^* = \begin{Bmatrix}T\\T\\T\end{Bmatrix} \equiv \begin{Bmatrix}T\\T\\T\end{Bmatrix}^*,$$

$$\begin{Bmatrix}A\\G\\A\end{Bmatrix} \equiv \begin{Bmatrix}A\\G\\A\end{Bmatrix}^* = \begin{Bmatrix}A^*\\G^*\\A^*\end{Bmatrix} \equiv \begin{Bmatrix}T\\C\\T\end{Bmatrix}$$

and so on.

Thus of the 16 combinations we obtain 8 pairs, the components of the pairs being identical with each other (in the above-mentioned sense). The remaining 48 cells have such a structure that equality $\alpha^{-1}=\alpha$ does not occur among them. Cell $\alpha=\begin{Bmatrix}A\\G\\T\end{Bmatrix}$ is not identical with cell $\begin{Bmatrix}T\\G\\A\end{Bmatrix}=\alpha^{-1}$. In precisely the same way cell $\alpha^*=\begin{Bmatrix}A\\G\\T\end{Bmatrix}^*=\begin{Bmatrix}T\\C\\A\end{Bmatrix}$ is not identical with cell $\begin{Bmatrix}T\\G\\A\end{Bmatrix}^*=\begin{Bmatrix}A\\C\\T\end{Bmatrix}=(\alpha^{-1})^*$. However, if we apply postulates I and II, we shall be obliged to consider that cells of type α and α^*, and also α^{-1} and $(\alpha^{-1})^*$ coincide in accordance with postulate I and α and α^{-1}, and also α^* and $(\alpha^*)^{-1}$ coincide in accordance with postulate II.

In this case the four cells in the example given below bear the same genetic load:

$$\begin{Bmatrix}A\\G\\T\end{Bmatrix}, \quad \begin{Bmatrix}T\\C\\A\end{Bmatrix}, \quad \begin{Bmatrix}T\\G\\A\end{Bmatrix}, \quad \begin{Bmatrix}A\\C\\T\end{Bmatrix}.$$

In this same case, the first cell is identical to the second and the third to the fourth on the basis of postulate I

$$\begin{Bmatrix} T \\ C \\ A \end{Bmatrix} \equiv \begin{Bmatrix} T \\ C \\ A \end{Bmatrix}^{*} = \begin{Bmatrix} A \\ G \\ T \end{Bmatrix} \text{ and } \begin{Bmatrix} A \\ C \\ T \end{Bmatrix} \equiv \begin{Bmatrix} A \\ C \\ T \end{Bmatrix}^{*} = \begin{Bmatrix} T \\ G \\ A \end{Bmatrix}.$$

The first and third cells, like the second and fourth, are identical in respect of postulate II, since

$$\begin{Bmatrix} A \\ G \\ T \end{Bmatrix} \equiv \begin{Bmatrix} A \\ G \\ T \end{Bmatrix}^{-1} = \begin{Bmatrix} T \\ G \\ A \end{Bmatrix} \text{ and } \begin{Bmatrix} T \\ C \\ A \end{Bmatrix} \equiv \begin{Bmatrix} T \\ C \\ A \end{Bmatrix}^{-1} = \begin{Bmatrix} A \\ C \\ T \end{Bmatrix}.$$

In such a case they are genetically identical and

$$\begin{Bmatrix} T \\ C \\ A \end{Bmatrix} \equiv \begin{Bmatrix} T \\ C \\ A \end{Bmatrix}^{*} = \begin{Bmatrix} A \\ G \\ T \end{Bmatrix} \equiv \begin{Bmatrix} A \\ G \\ T \end{Bmatrix}^{-1} = \begin{Bmatrix} T \\ G \\ A \end{Bmatrix} \equiv \begin{Bmatrix} T \\ G \\ A \end{Bmatrix}^{*} = \begin{Bmatrix} A \\ C \\ T \end{Bmatrix} \equiv \begin{Bmatrix} A \\ C \\ T \end{Bmatrix}^{-1} = \begin{Bmatrix} T \\ C \\ A \end{Bmatrix}.$$

Consequently 48 combinations of bases in cells with 3 elements give 12 essentially different combinations, each of which will be four-fold degenerate.

We have, therefore, altogether 20 essentially differing cells with a different genetic load. The problem of individualization of each cell, i.e. the problem of which cell each amino acid corresponds to, remains unsolved. It is now obvious that the 20 "letters" are not equal among themselves. Eight of them have two-fold and 12 have four-fold degeneracy. This already signifies some degree of structure in the building up of the amino acid chains, since the entropy of "communication" [7] with different degeneration of the "letters" is less than in the case if all 64 possible cells determined the individual "letters" of the "genetic alphabet". Actually:

$$H_{\text{DNA}} = -\sum_{i=1}^{8} p_i \log p_i - \sum_{j=1}^{12} - p'_j \log_2 p'_j = -\frac{8}{32}\log_2\frac{1}{32} - \frac{12}{16}\log_2\frac{1}{16}$$

$$= \frac{17}{4} < -\sum_{k=1}^{64} p''_k \log_2 p''_k = \log_2 64 = 6 \text{ twin units} = H^0.$$

It remained to account for the physical meaning of postulates I and II. Let us begin with postulate II. In view of the symmetry of DNA in relation to the direction of winding of the spirals of the saccharo-phosphate groups, we cannot assume that in the structure of "pure" DNA (without amino acids and bases) an upward or downward direction can be distinguished in any way. In this connexion it is difficult to suppose that an amino acid, combined with 3 bases in the order $\begin{Bmatrix} A \\ T \\ G \end{Bmatrix}$, will be distinguishable from an amino acid combined with bases arranged in the order $\begin{Bmatrix} G \\ T \\ A \end{Bmatrix}$. If this difference did exist, the form of the amino acid would depend on how we "write" the bases—from above downward or from below upward. There are now no causes of a physico-chemical character which would prevent us from accepting a comparatively simple postulate, II, signifying only that the same amino

acid is combined in DNA in an identical fashion if in it there are encountered at two elevations combinations of bases:

$$\begin{Bmatrix} A \\ G \\ C \end{Bmatrix} \text{ and } \begin{Bmatrix} C \\ G \\ A \end{Bmatrix} = \begin{Bmatrix} A \\ G \\ C \end{Bmatrix}^{-1}.$$

Let us turn to postulate I. It is a well-known fact that A attaches to T, and G to C, suggesting that the right column, entirely composed of bases united to the bases of the left column, must as a whole bear the same "genetic information" as the left column. This follows from the fact that the appearance of T at the level of base A is an authentic event and not determined by probability. Events with a probability of unity bear no additional information. This position may spread also to bases inside the given cell.

From what has been said it is impossible to make a simple conclusion on how many bases take part in the formation of one amino acid: three or six. Actually, cells α and α^* may also satisfy postulates I and II jointly, as well as separately. Thus the same amino acid may be attached both to the cell

$$\alpha = \begin{Bmatrix} A \\ G \\ C \end{Bmatrix},$$

and to the twin cell (α, α^*), i.e. to

$$\begin{Bmatrix} A|T \\ G|C \\ C|G \end{Bmatrix}.$$

It is clear that the cell (α, α^*) is in no way distinguishable from the twin (α^*, α) in accordance with postulate I.

We leave unsolved the question: does attachment of amino acids take place to pairs of combined cells (α, α^*) or do two similar amino acids attach to identical (in the sense of postulate I) cells (α) and (α^*).

Translated by B. HAIGH

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DETERMINATION OF CERTAIN MOLECULAR PARAMETERS OF DEOXYRIBOSENUCLEIC ACID BY THE METHOD OF VISCOMETRY*

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IN a previous paper [1] we gave our conclusion regarding the relationships between the molecular weight (M) and the intrinsic† viscosity [η] for molecules of deoxyribosenucleic acid (DNA). In the range of molecular weights from 3×10^6–6×10^6 this relationship holds good, but at lower and higher values of the molecular weight a discrepancy is observed between the molecular weights of DNA determined by light scattering and by calculation from the value of [η]. This discrepancy is explained, in all probability, by the fact that the empirical correction which we introduced [1] gives a value α in the relationship $[\eta]=KM^{\alpha}$ which is closer for polynucleotide chains than for the double spiral molecule of DNA [2]. In addition, in calculating the size of the segment of DNA we set out from a solitary polynucleotide chain. It is natural that the double spiral molecule of DNA should be more rigid [3] with a correspondingly larger segment. In fact, calculation of the size of the segment from the relationship $R^2/h^2=a$, by calculation of the free rotation of the double spiral structure of DNA, gives a minimum number of nucleotides in a segment of the molecule of DNA (a) of the order of 140–160. It has to be pointed out that the magnitude of the angle of internal rotation used in these calculations was very approximate [1, 4] since up to the present time there have been no indications in the literature [3] concerning bonds rotations along the chain of the main valencies of the molecule of DNA.

Roughly the same number of nucleotides (80–150) in the DNA segment is found by calculation from the assumption that on the average the ratio of the maximum length of the chain to its most probable extension, independently of variations in the internal structures of the linear macromolecule, is equal to $0{\cdot}5\,N^{\frac{1}{2}}$ (where N in our case amounts to half the degree of polymerization of DNA) [6].

We obtain values of the same order from studies of the molecules of DNA and DNP. In this case, judging from our own findings and those in the literature [3], and in consequence of the relatively high elastic deformation of DNP [3, 7–11], it has to be considered that between the protein molecules present on DNA there are definite interspaces around which internal rotation along the chain of the main

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† In accordance with the recommendations of the Commission on Macromolecules of the International Union of Theoretical and Applied Chemistry the term "intrinsic viscosity" must be replaced by the term "limiting viscosity number". In the present paper we introduce this term [5].

valencies of the DNA molecule is effected. Since the quantity of low molecular weight histone permits its arrangement on the DNA molecule in the full length of its extended β-configuration [3], and considering that the molecular weight of histone is of the order of 9000 [3], and that the average molecular weight of the amino acids is 125, we may conclude that one molecule of a given protein, disposed on DNA, corresponds geometrically to 36 nucleotides (9000/125=72). This value is small in comparison with the value for a segment given above. Hence it may be concluded that between the links in the DNA molecule around which rotation takes place, there is room for at least 4 molecules of low molecular weight histone, equivalent in geometrical space to 144 nucleotides. In addition, the findings of a series of investigations [12, 13] that the energy of activation of DNA is 60–93 kcal allows calculation, from the relationship $U/RT=a$ [1], of the size of the DNA segment (in calculation on a single polynucleotide chain) to be 100–156 nucleotides, irrespective of their molecular weight, since the above mentioned size of the potential barrier is determined solely by the energy of interaction of neighbouring radicals and by the type of bond. It is true that the size may vary during transition from one solvent to another.

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Finally, it should be mentioned that immediately after publication of our work [1] the problem of the DNA segments was raised in the paper by Rice and Doty [14], based on experimental determinations of the magnitude of the energy of activation during denaturation of DNA molecules. For specimens of DNA obtained by different methods but from the same source the energy of activation lies within the range 35–93 kcal. During denaturation of DNA, rupture occurs of the hydrogen bond between the bases. In comparison with other systems connected by H-bonds it is clear that the energy of activation of such reactions must lie within the range of 1–10 kcal per pair of bases. If we consider that in a molecule of DNA there are roughly 10,000 pairs of bases then the magnitude of the energy of activation of the whole molecule must be given a value of 10^4–10^5 kcal, which is many times greater than the experimental results. In consequence of this, the authors of the papers cited consider that the process of denaturation of DNA in its initial stage affects only one segment, consisting of 10–100 pairs of bases, and it is to be expected that a segment with 10–50 pairs of bases belongs to a molecule with H-bonds which are less stable or are destroyed by various causes, while a segment of 50–100 pairs of bases belongs to native DNA.

Thus we see that different methods of determination of the size of the segment of the DNA molecule permit its value to be from 130 to 160 nucleotides, which agrees roughly with the size of the minimum chemical unit of DNA [3]. For an accurately calculated value of the size of the segment we accept 144 nucleotides, which corresponds to the geometrical placing of 4 molecules of low molecular weight histone (or of 1 molecule of a histone with $M=3500$), situated along the whole length of the extended β-configuration.

In this case, by analogy with the previous communication [1], considering purely geometrically an ideal polynucleotide chain with segments corresponding to the actual double spiral molecule of DNA, we have

$$[\eta] = \varphi(R^2)^{\frac{3}{2}}/Mq_{\Theta} \qquad (1)$$

where $\varphi = \text{const} = 2{\cdot}1 \times 10^{21}$, and since $N_c = M/M_c$ where N_c is the degree of polymerization of the segments with molecular weight M_c, and $R^2 = N_c b^2$, where b is the length of the segment, we finally obtain:

$$M = \frac{[\eta]^2 M_c^3 q_\varphi^2}{\varphi^2 b^6}.$$

The polydispersion factor q_φ may be estimated in size as 1·95, since the polydispersion of DNA obeys the relationship $M_n : M_w : M_z = 1 : 2 : 3$ [15], and then:

$$[\eta] = 40 \times 10^{-4} M^{\frac{1}{2}}. \tag{2}$$

However, according to Flory [16] the value of α in the expression $[\eta] = KM^\alpha$, equal to 0·5, corresponds to the θ-point, the physical meaning of which consists in the fact that at a certain temperature θ (Flory's point) the interaction of the individual segments of the chain in an inactive solvent does not affect the dimensions of the molecule, and this has its most compact presentation.

For solutions not at Flory's θ-point [16]

$$[\eta] = K_1 M^{\frac{1}{2}} \beta^3 = KM^\alpha. \tag{3}$$

Since the value $[\eta]$ has the dimension of volume per unit weight and is proportional to the effective volume of the isolated molecule in solution, divided by its molecular weight, the value β^3 may be regarded as a factor of increase in volume of a dispaced molecule with an unchangeable mean value of the square of the distance between the ends of the molecule R^2 and β as the corresponding coefficient of "longitudinal expansion" of the molecules, given by

$$\beta^3 = \frac{[\eta]}{[\eta]_\theta} = \frac{(R^2)^{\frac{3}{2}}}{(R^2_0)^{\frac{3}{2}}}$$

where $[\eta]_\theta$ is the limiting viscosity number (LVN) at the θ-point. However, as seems perfectly convincing to us, a molecule, consisting of several segments, has an extended configuration even at the θ-point and, generally speaking, in such a molecule the ability to change the configuration must be expressed extremely feebly. Naturally in this case straight forms, corresponding to the logarithmic form of the equations $[\eta] = K_1 M^{\frac{1}{2}} \beta^3$ (the latter corresponds to the equation $[\eta] = KM^\alpha$) and $[\eta] = K_1 M^{\frac{1}{2}}$, must be transformed at some S-point. We may estimate the size of this portion of DNA which is incapable of changing its configuration during transition at the θ-point as of the order of two segments of DNA [3], which corresponds to a value of M of 200,000. This value coincides with the molecular weight of Kuhn's element for the macromolecular chain of DNA, calculated by the theory of light scattering [15]. Consequently the LVN of DNA at the S-point, according to formula (2) is 1·8. From reports in the literature [15, 17–20] (α=0·93; 0·93; 1·0; 1·08; 1·00), the value of α for DNA can be estimated to be of the order of 1, which is perfectly natural for rigid, asymmetrical molecules. Transferring equation (2) into a logarithmic form and knowing the value of α in equation (3), we may find the numerical solution of this equation for a known S-point and angle α:

$$[\eta] = 9{\cdot}0 \times 10^{-6} M. \tag{4}$$

Thus from the expression (4) it is seen that the relation $[\eta] = f(M)$ for DNA is calculated by Staudinger's equation.

Combining equations (1) and (4) (at $q_\varphi=1$), we obtain a relationship between the mean squares of the distances and M and $[\eta]$ as follows:

$$\sqrt{R^2} = 0{\cdot}162\, M^{\frac{1}{2}} \tag{5}$$

and

$$\sqrt{R^2} = 376[\eta]^{\frac{1}{3}}. \tag{6}$$

In several papers[21, 22] it was noted that there are in existence two types of DNA molecule—C_1 and C_2 with the relationship $[\eta]=f(M)$. The results obtained apply to DNA type C_1, which includes the majority of nucleic acids examined. Type C_2 DNA, it appears to us, is a preformed type C_1. We deal with this point in detail elsewhere [23].

As can be seen from Table 1, our suggested formulae (4), (5) and (6) are fully justified by comparison of the values of M, R^2 and $[\eta]$, determined by independent methods, and are actually in the range of molecular weights for DNA of $(0{\cdot}1\text{–}10)\times 10^6$.

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TABLE 1. THE DEFINITION OF CERTAIN MOLECULAR PARAMETERS OF DNA BY VISCOMETRY

Reference in literature	Molecular weight ($\times 10^{-6}$)		Mean square of distance between ends of molecule $\sqrt{R^2}$ (Å)		Limiting viscosity number $[\eta]$ (100 cm³/g)		Asymmetry of molecules a/b		
	From the literature	By formula (4)	From the literature	By formula (6)	From the literature	By formula (4)	By Kuhn	By Polson	By formula (10)
[17]	0·48*	0·45	—	995	4·0	4·3	112	89	97·5
[17]	0·56*	0·50	—	1100	4·5	5·0	120	96	105
[17]	0·60*	0·66	—	1150	5·6	5·4	125	100	109
[17]	0·97*	0·98	—	1580	8·9	8·75	159	127	139
[1]	2·5	—	4000	3000	—	22·5	256	204	223
[1]	3·5	3·42	4000	3740	30·7	31·4	310	242	263
[24]	3·5	3·45	—	3740	31·0	31·5	311	243	264
[24]	4·2	4·22	4730	4220	38·0	37·8	322	264	289
[1]	4·4	—	—	4350	—	39·6	336	271	295
[21]	4·6	4·78	4990	4770	43·0	41·5	347	277	303
[1]	4·65	—	4480	4500	—	42·0	350	279	305
[1]	4·7	—	—	4530	—	42·3	351	280	306
[17]	5·0*	4·6	—	4730	41	45	362	289	315
[1]	5·8	5·65	—	5200	50·6	52·1	389	310	339
[14]	5·85	5·88	—	5250	53	52·6	391	312	340
[1]	5·85	5·93	4950	5250	53·4	52·6	391	312	340
[1]	5·9	5·7	5400	5280	51·0	53·1	394	314	343
[21]	6·0	5·92	—	5350	53·3	54	396	316	345
[25]	6·18	—	5230	5425	—	54·7	398	318	347
[25]	6·47	—	5480	5530	—	58·2	411	328	359
[25]	6·48	—	5410	5570	—	58·3	411	328	360
[14]	6·6	6·8	—	5650	62	59·4	416	332	362
[1]	6·85	—	5030	5800	—	61·6	425	338	368
[1]	6·87	—	5030	5820	—	61·8	426	339	369
[1]	7·7	—	7100	6300	—	69·3	449	368	391
[22]	7·7	6·7	—	6300	60·0	69·3	449	358	391
[24]	7·7	8·0	—	6300	72	69·5	450	359	392
[24]	7·7	8·0	—	6300	72	69·5	450	359	392
[26]	8·2±2	7·8	—	6600	70±2	73·8	463	370	403
[14]	8·2	8·0	—	6600	72	73·8	463	370	403

* Mean value calculated by the equations of Perrin and Mendelkorn-Flory [17].

In addition to what has been stated, it appears possible to us to evolve a simpler expression for calculation of the asymmetry of the molecules of high molecular weight compounds, and in particular DNA. Actually, substituting the value M from expression (1) (at $q_\varphi=1$) in the known relationship $\frac{M\bar{v}}{N}=\frac{4}{3}\pi ab^2$, where $\bar{v}$ is the specific partial volume of the molecule of the polymer (equal to 0·55 for DNA [27]), N is Avogadro's number, and a and b are the major and minor semi-axes of the molecule respectively, we have:

$$\frac{(R^2)^{\frac{3}{2}}\varphi\bar{v}}{[\eta]N} = \frac{4}{3}\pi ab^2. \tag{7}$$

However, if we consider that $a/b \gg$ twice the radius of rotation (R_g) of an equivalent ellipsoid of rotation roughly equal to $a/\sqrt{5}$, and that for an irregularly curved chain it is equal to $(R^2/6)^{\frac{1}{2}}$, we may write

$$a \approx \sqrt{R^2}, \tag{8}$$

which is fully confirmed by experiment [25]. Substituting from (8) the value $\sqrt{R^2}$ in the expression (7), we obtain:

$$\frac{a}{b}=\sqrt{\frac{4\pi N[\eta]}{3\varphi\bar{v}}},$$

i.e.

$$\frac{a}{b}=34{\cdot}7\sqrt{\frac{[\eta]}{\bar{v}}} \tag{9}$$

with $a/b \gg 2$ and for DNA:

$$a/b = 47\sqrt{[\eta]} \tag{10}$$

with $a/b \gg 2$.

The values of $[\eta]$ (2·4–6·9 and 10) are given in 100 cm^3/g.

The magnitudes of the asymmetry of the molecule of DNA, calculated from formula (10), agree well with the corresponding parameters obtained by Kuhn's formula [28] and Polson's formula [29] (Table 1), and also are in agreement with the calculated values obtained with Simha's equation [30], by calculation that 1 g of dry Na salt of DNA combines with 0·35 g of H_2O [31], i.e. that the increment of viscosity (γ) must increase 1·64 times.

Analysis of formula (10) permits the following conclusion to be drawn. In a number of papers it was shown [10, 11, 26] that the viscosity of DNP is roughly one-half that of the corresponding viscosity of DNA. In this case, considering that the asymmetry of DNP is mainly due to the DNA which enters into its composition [23], the DNP molecule is shortened two-fold in relation to the DNA molecule, i.e. by approximately 30 per cent. On the other hand it is known that DNA molecules may be found in two configurations: a crystalline A and a paracrystalline B form, and moreover the former is 30 per cent shorter than the latter and is formed in quite a low relative humidity (of the order of 75 per cent). If the projection of the length of the nucleotide in its B configuration is 3·4 Å, in the A configuration it is equal to 2·55 Å. It is believed [32] that proteins are combined with DNA in the B configuration, since according to Astbury [33] a stereochemical relationship is observed in the distances between the lateral groups in the protein and the nucleotides in the DNA (at 3·4 Å).

However, from our point of view there are other and no less interesting relationships. The characteristic period of proteins in the α-configuration—5·1 Å—corresponds exactly to the distance apart of the two nucleotides along the axis of the DNA molecule in the *A*-configuration—5·1 Å.

It may be thought that DNA, existing in the *B*-configuration, opens out proteins to the α-configuration and combines with them. Meanwhile the protein dehydrates the DNA, as a result of which it becomes compressed to the *A*-configuration; the DNA, in its turn, elongates proteins to the α-configuration, and here again is observed a remarkable stereochemical relationship: the dipeptide of the protein is 5·1 Å and the dinucleotide is also 5·1 Å. Thus the transformation of DNA→DNP corresponds to the transformation of DNA from the *B* to the *A* configuration, and of protein from the β- to the α-configuration. This transformation is accompanied by a 30 per cent shortening of the DNA molecule. Evidently for the corresponding dehydration of DNA it is necessary to have sufficient (18 per cent) of protein compared with the weight of DNP, since inversion of DNA into DNP is observed [3].

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In conclusion we consider it necessary to point out that the viscosity must be measured at an initial concentration of DNA of ~0·004 per cent in a 0·2 M solution of NaCl in order to exclude interaction of the molecules (theoretically this is excluded at a DNA concentration of 0·0028 per cent [25]), to remove the electroviscosity effect which appears with the presence in the DNA molecule of considerable free charge in a solution of low ionic strength, and also for other considerations which are discussed in our previous paper [1].

SUMMARY

(1) The size of the segment of the DNA molecule has been calculated by four independent methods.

(2) Relationships have been worked out for DNA between the limiting viscosity number and the molecular weight (4), the mean square of the distance between the ends of the molecule and the molecular weight (5) and the viscosity (6) respectively.

(3) An expression has been found to calculate the asymmetry of molecules of compounds of high molecular weight (in which $a/b \gg 2$) (9) and in particular for DNA (10).

(4) It has been shown from experimental and mathematical data that there are stereochemical relationships between DNA in the *A*- and protein in the α-configurations, and the hypothesis is expressed that in DNP the DNA and proteins are found in the *A*- and α-configurations respectively.

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THE CHANGES IN THE ULTRA-VIOLET ABSORPTION SPECTRUM OF DISSOLVED MYOSIN ON DENATURATION*

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THE absorption spectra of protein solutions at pH 9 in the 248–320 mμ region are unaltered by denaturation if oxidation and decomposition are prevented. In some cases the absorption increases uniformly throughout the ultra-violet because the coagulation causes an increase in light scattering [1]. An exception occurs with the irreversible rise in absorption at $\lambda < 280$ mμ, which is found, for example, with ribonuclease acidified after alkali denaturation [2].

Pure myosin behaves in an unusual way in this respect. Inactivation by heat, $CdCl_2$, pH 5 (or pH < 11·0), causes a considerable rise in the true absorption at $\lambda < 265$ mμ. This effect may be related to the extreme heat sensitivity, and to the marked susceptibility to structural or configurational changes. The data we give for

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the changes produced by inactivation are interesting in comparison with those for other proteins.

Pure actin-free myosin was prepared from rabbit muscle by Szent-Gyorgyi's method, as modified by Lyubimova, and its ultra-violet absorption curve was measured at 1–2 mμ intervals on an SF-4 spectrophotometer in a 1 cm path at 0·5 mg/ml in 0·5 M KCl. The pH was fixed by adding either 0·2 M borate buffer (0·05 M in the working solution), or by adding 0·2 M HCl or 0·2 M KOH. The pH was measured potentiometrically (glass electrode, LP-5 potentiometer).

The curve for 0·51 mg/ml at pH 8 (Fig. 1) shows the main bands of phenylalanine at 258 and 264 mμ, of tyrosine at 268 and 282 mμ, and of tryptophane at 290–1 mμ, with a maximum at 276–8 mμ and a minimum at 250–1 mμ. The ratio $K=E_{276}/E_{250}$ was 1·82 (uncorrected for scatter). The spectrum changed after standing the solution for 10–12 days in the refrigerator, although the ATPase activity was retained; the

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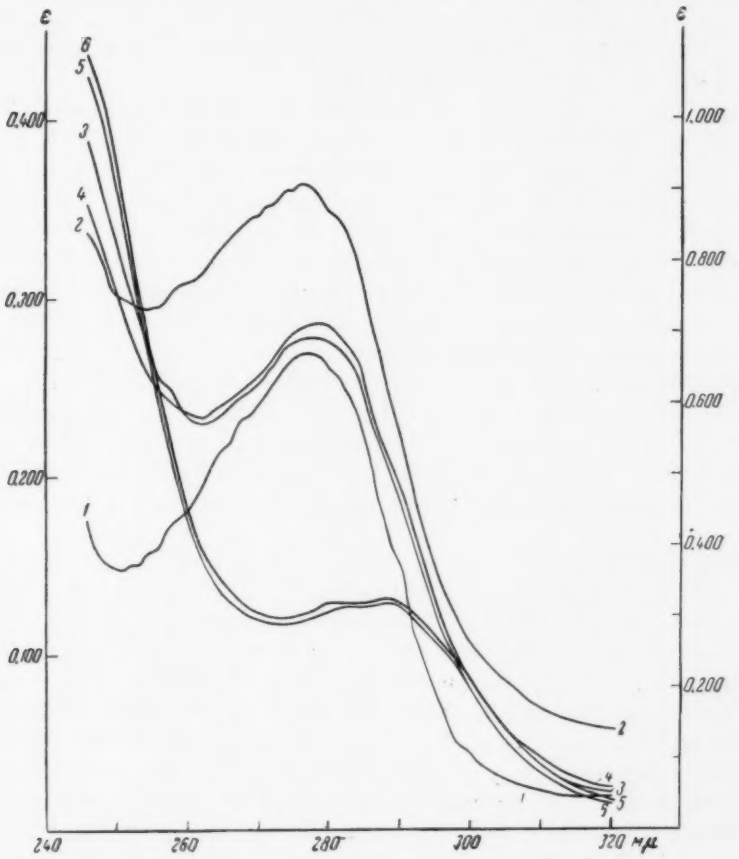

FIG. 1. Effect of elevated temperatures on the ultra-violet absorption of myosin. (1) and (2) are for 0·51 mg/ml and pH 8·0; (3) and (4) for 0·42 mg/ml, pH 9·95; (5) and (6) for 0·42 mg/ml, pH 12·5 (and are read on the scale on the right). (1), (3) and (5) are for the active protein; (2), (4) and (6) for that inactivated at 37°C for 6 hr.

absorption at $\lambda < 260$ mμ increased, and K fell to 1·6–1·7 (partly due to increase in the scattering), while the minimum shifted to 250 mμ.

On keeping the solution at 37°C for 1 hr (which inactivates the myosin), similar but more marked changes were found (see Fig. 1). The minimum shifted to 255–6 mμ, and certain fine-structure bands, particularly at 258 mμ (Fig. 1, curve 2), appeared. Similar changes have been found on raising the pH to 10 [3]. As the pH of a myosin solution inactivated by heating is raised the increase in absorption diminishes (above pH 9·2), and virtually vanishes above pH 10 (Fig. 1, curves 3–6). This is clearly not related to the increased absorption at $\lambda < 275$ mμ or 300 mμ at pH 12 found with, for example, egg albumin or ribonuclease. In these latter proteins the effect is related to rupture of some of the H-bonds on tyrosine groups, and to ionization of these groups at pH 11·5–12, which shifts the 275 mμ bond to 290 mμ. No such increase occurs with myosin, which might therefore be said to be a protein with its tyrosine groups free in the native state, like serum albumin, chymotrypsin and lysozyme. However, myosin differs from these proteins in denaturing at a comparatively low alkaline pH (10·5–11·0; see below), so the tyrosine groups must be free at pH $\geqslant$ 11·0, no matter what their state in the native protein.

The reduction in K on thermal inactivation is largely caused by the increase in light scattering. It is important to know whether the true absorption increases also; to this end scattering corrections were calculated for 252 and 276 mμ by measuring the optical densities at 320 and 360 mμ, and using Rayleigh's law for the scattering loss $\varepsilon_\tau = k\lambda^{-n}$. The true absorption was assumed negligible above 320 mμ, and k and n were taken to be constant over the 250–360 mμ range. This assumption is based on the results of Schauenstein and Bayzer [4], which showed k and n to be constant over a wide range, while ε_τ and the true absorption were additive (for serum albumin). For myosin we found n in each experiment as $(\log \varepsilon_{320} - \log \varepsilon_{360})/(\log_{360} - \log_{320})$, and then corrected for ε_τ at 252 mμ (and 276 mμ) from $\varepsilon_\tau/\varepsilon_{320} = (320/252)^n$.

The K for the true absorption of the active protein, K_u, was found to be 1·98 (mean for 14 myosin preparations). On standing at 2–4°C for 10–14 days the value fell (lowest value found 1·73). For the freshly-prepared protein n varied from 3·44 to 4·0, falling to 2·5–3·0 for 14 day material; this indicates that the particles increase in size.

Heating to 37°C for 1·5–6 hr, or to 90–95°C for 10 min causes a large increase in the true absorption at 252 mμ (denoted by $\Delta\varepsilon_{252}$, as a percentage of the initial value), and a small one ($\Delta\varepsilon_{276}$) at 276 mμ. The means for 9 solutions were 23·2 $\pm$ 3·2 per cent and 5·1 per cent respectively. When old solutions of low n (3·0 or less) were heated, $\Delta\varepsilon_{252}$ was only half the above mean value; when the fresh protein is heated and aggregated (ageing products, i.e. large particles, absent) the effect was maximal.

In one series $\Delta\varepsilon_{252}$, K_u, $\Delta\varepsilon_{320}$, K, ε_{252} and the ATPase activity were all determined on the same solution (see Fig. 2). The rise in $\Delta\varepsilon_{252}$ lags behind the activity change, and the rise in ε_{320} is also delayed, but less so. When an active myosin solution of concentration 0·5 mg/ml in borate buffer at pH 8·0 was kept for 1 hr at 37°C with $7{\cdot}4 \times 10^{-6}$ M ATP, the true and total absorptions in the 250–360 mμ range remained constant. If the ATP content was lower $\Delta\varepsilon_{252}$ rose. The least ATP content effective in preventing the change was under 12·5 molecules of ATP per molecule of myosin (taking the molecular weight of the latter as 850,000).

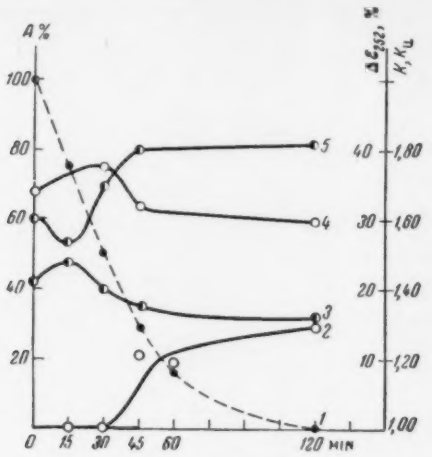

FIG. 2. Changes in activity and absorption during heating at 37°C. Concentration during heating 4 mg/ml, during absorption measurements 0·5 mg/ml, pH 8·26. (1) percentage activity, (2) $\Delta\varepsilon_{252}$; (3) $K=\varepsilon_{206}/\varepsilon_{252}$; (4) K_u; (5) absorption at 320 mμ, ε_{320}.

Using myosin in 0·1 M guanidine carbonate (brought to pH 8·6 with H_2SO_4) we found that n was greatly increased, the apparent rise in density at 252 mμ being very small, while $\Delta\varepsilon_{252}$ was negative (so K_u increased). The activity fell by 23 per cent. Although K_u and n were not affected by heating, while the true absorption at 252 mμ fell, the apparent absorption rose by almost a factor of 3 (see Table 1). Hence the guanidine reduces the true absorption while inactivating the protein, which corresponds to depolymerization (n increased). The guanidine prevents any rise in true absorption on heating, but, unlike ATP, does not prevent the rise in apparent absorption.

If the KCl content was reduced to 0·125 M (i.e. to the point where myosin begins to precipitate), the light-scattering increased greatly and the true absorption slightly. After incomplete (partially reversible) acid inactivation at pH 5·5, followed by rapid return to pH 6·2 the $\Delta\varepsilon_{252}$ was less than half the value found on thermal inactivation, and slightly exceeded it after heating. Complete acid inactivation gave a large $\Delta\varepsilon_{252}$, which fell somewhat after heating. Complete inactivation in $2{\cdot}4\times10^{-4}$ M $CdCl_2$ gave about the same $\Delta\varepsilon_{252}$; here only a minute increase in scattering occurred, and the rise in true absorption can be seen directly (Fig. 3). Again $\Delta\varepsilon_{252}$ fell on heating. Thermal inactivation in 0·001 M cysteine gave a $\Delta\varepsilon_{252}$ close to the maximum found.

The spectral changes at alkaline pH $\leqslant$ 10·5 are completely reversible, but become irreversible at high pH (increase in short-wave absorption, see Fig. 3), and comparable with those found with $CdCl_2$. The same irreversible spectral changes in alkali have been found with ribonuclease [2], and have been supposed related to alkali denaturation. The $\Delta\varepsilon_{252}$ produced by alkali is the highest of all, the next being thermal inactivation with cysteine present, and then that for the solution brought to pH 10·75, neutralized, and heated ($\Delta\varepsilon_{252}=45$ per cent). Fig. 4 shows how $\Delta\varepsilon_{252}$ depends on the highest pH attained before neutralization; irreversible changes begin at pH 11·0,

TABLE 1. CHANGES IN THE ABSORPTION OF MYOSIN UNDER VARIOUS CONDITIONS

Solution	$\Delta\varepsilon_{252}$ (%)	ε_{τ_0} (%)	ε_{τ} (%)	K_u^0	K_u	n_0	n
In 0·125 M KCl Control in 0·5 M KCl	4·9	14·3	58·4	1·94	1·83	3·34	3·08
Acidified to pH 5·5 and returned to pH 6·2	10·0	38·9	59·8	1·90	1·69	3·67	3·88
The same, followed by thermal denaturation	27·1	38·9	60·6	1·90	1·53	3·67	4·0
In $2{\cdot}4\times10^{-4}$ M $CdCl_2$ and 0·5 M KCl. Control without $CdCl_2$	35·0	11·5	17·0	1·73	1·22	3·44	3·44
The same, followed by thermal denaturation	20·5	11·5	28·0	1·73	1·32	3·44	3·79
1 hr in the cold at pH 5·0 followed by return to pH 8·8	31·4	22·2	50·9	1·82	1·38	2·55	3·44
The same, followed by thermal denaturation	25·8	22·2	58·4	1·82	1·54	2·55	3·79
In 0·1 M guanidine and 0·5 M KCl. Control without guanidine	−16·9	20·2	23·0	1·82	2·00	2·59	4·0
The same, followed by thermal denaturation. Control with guanidine	−7·1	23·0	62·8	2·00	1·98	4·0	4·0
Thermal denaturation of a control solution in 0·5 M KCl and 0·001 M cysteine	42·7	23·5	42·5	1·95	1·43	2·26	3·44

Notes: $\Delta\varepsilon_{252}$ and $\Delta\varepsilon_{276}$ are the increases in true absorption at 252 and 276 mμ as percentages of the values for the control. ε_{τ} and ε_{τ_0} are the fractions of the apparent absorption at 252 mμ (as percentages of the totals) for the experimental and control solutions respectively. K_u and K_u^0 are the $\varepsilon_{276}/\varepsilon_{252}$ ratios for the experimental and control solutions respectively; n and n_0 are the indices in the Rayleigh scattering formula. Unless otherwise indicated the control solutions are always 0·5 mg/ml of myosin in 0·5 M KCl.

and this might be taken as alkali denaturation, which is, however, known to be irreversible at pH 10·0 [5]. Hence a myosin solution brought to pH 10–10·6 and then neutralized is inactivated without spectral change, which then gives a large $\Delta\varepsilon_{252}$ on heating. The spectral and denaturation effects of alkali have clearly to be differentiated.

It is natural to suppose that this effect has the same causes as in thermal, acid and $CdCl_2$ inactivation. This was proved to be the case as follows. A solution at 0·64 mg/ml in 0·5 M KCl (pH 6·5) was inactivated by heating to 98°C for 10 min. The solution was then brought to pH 11·56, and then to pH 6·5 after 30 min. The absorptions of this solution and of a control (heat inactivated, but with a neutral mixture of acid and alkali added) coincided precisely over the whole 246–320 mμ range.

In another series followed by heating, myosin solutions were brought to pH 11·63 and then neutralized. The apparent absorption was increased by the heating, but

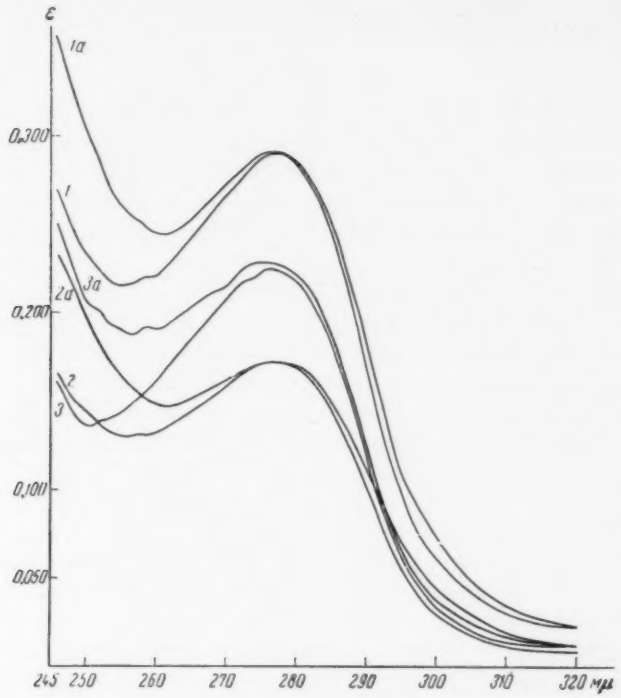

FIG. 3. Effects of $CdCl_2$ and alkali on the ultra-violet absorption of myosin: (1) 0·4 mg/ml, pH 8·83; (1a) 0·4 mg/ml, pH 8·83, after 30 min at pH 11·63; (2) 0·49 mg/ml, pH 8·60; (2a) 0·49 mg/ml, pH 8·65 after 30 min at pH 11·13; (3) 0·38 mg/ml, pH 8·0; (3a) 0·38 mg/ml, pH 8·0 in $2{\cdot}4 \times 10^{-4}$ M $CdCl_2$. The values of ε are reduced by factors of 2 in curves (2) and (2a).

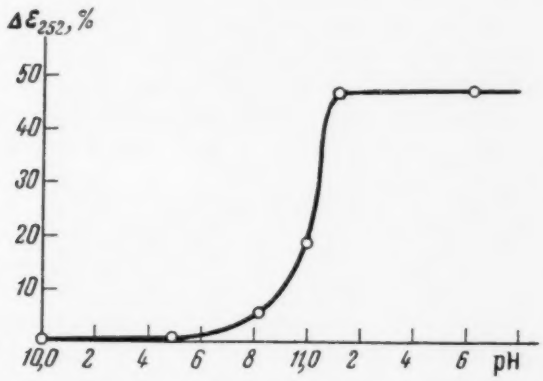

FIG. 4. Irreversible increase in absorption of dissolved myosin vs. pH. (Myosin kept for 30 min at alkaline pH and then neutralized.)

$\Delta\varepsilon_{252}$ was reduced. Hence thermally inactivated myosin gives no further spectral change with alkali, just as with alkali-inactivated solutions, which give no change on heating.

DISCUSSION

The main result is that there is a precisely defined increase in the true absorption of myosin inactivated in a pH range where the protein is stable. If corrections for scattering are applied using Rayleigh's formula for the highly asymmetric particles of myosin, a doubt as to the accuracy must arise. Errors may be caused by assuming k and n constant; another large source of error lies in the measurement of the small absorption at 360 mμ.

These errors are ruled out if the rise in absorption and scattering at 320–360 mμ is negligible, as occurs when alkali and $CdCl_2$ are used. Acid and heating produce an invariable increase in the true absorption at 252 mμ only with the active protein. The good reproducibility (5 of the 17 preparations gave a small $\Delta\varepsilon_{252}$ (11 per cent) and the others on average of 23 per cent) indicates that such an effect occurs here. The lack of an extra rise in $\Delta\varepsilon_{252}$ on heating acid- or $CdCl_2$-inactivated protein shows that all three agents produce the same effect.

The last series of experiments also shows that this irreversible inactivation is identical with the spectral changes occurring in alkali denaturation at pH 11. Alkali inactivation at pH 10 gives no such changes. We may therefore suppose that the effect is related to the denaturing action, or to some post-denaturation changes. The fact that similar changes are only found with alkali with other proteins can be explained by saying that the alkali deforms the structures most strongly. Myosin gives the same effect with less energetic agents, which accords with its high lability.

We can only imagine what the structural changes may be. The effect is similar to two types of spectral change observed earlier with proteins and first studied by Schauenstein as an "extra" true absorption (unrelated to aromatic amino acids) appearing as a very broad band in the 250 mμ region in the spectra of gels from silk fibroin, collagen and actomyosin. This band is always present, but becomes stronger on stretching fibres or films, and on alkali treatment. The absorption was assumed specific to the protein structure and caused by the enol form of the peptide linkage, which occurs at pH 7 and predominates at pH 12. The structural disorganization ("disaggregation") reduces this enol absorption, while aggregation mainly increases it. It is supposed that the approach between adjacent areas of the chains in the extended form causes H-bridges to form between the chains, and thus accentuates the enol absorption; the process can be considered as a sort of aggregation in between chains [6].

We did not, however, find that a band appeared at 250 mμ, but the short-wave absorption showed an increase, similar to the increase in peptide-group absorption at 205 mμ in irreversible thermal denaturation in serum albumin. The effect is ascribed to changes in the positions of chain links which influence the peptide bond configuration [7]. It is related to the irreversible stage in denaturation, takes some while to develop, and passes through a maximum on prolonged heating. No light-scattering corrections were applied, but if we take these data as reliable, the additional absorption we found at 252 mμ could be the long-wave edge of a strengthened band with its maximum at 200–210 mμ.

This assumption of peptide group reconstruction as the cause of diffuse bands or continuous absorption still constitutes the sole attempt to explain these effects, although it is vague and has not been demonstrated. It would indicate that the true absorption in myosin is related to structure changes following destruction of the "secondary" and "tertiary" structures, i.e. when the mutual positions of the main peptide chains have been disturbed the chains can come closer together; this reconstruction can occur at the start of denaturation. The resulting aggregation can be followed from the fall in the birefringence (shortening or coiling of the particles) and from the inactivation. Fig. 5 shows the changes in the viscosity and birefringence

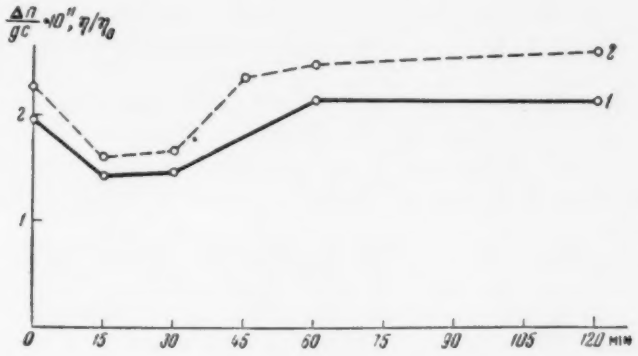

FIG. 5. Flow birefringence and relative viscosity at 37°C. Concentration 4 mg/ml. (1) flow birefringence; (2) viscosity.

at 37°C; these indicate aggregation and occur at the same time as the spectral changes in myosin solutions of the same concentration under the same conditions (Fig. 2). The rise in $\Delta\varepsilon_{252}$ is clearly delayed relative to the inactivation, to the fall in birefringence, and even to the increase in light-scattering (Figs. 2 and 5). Factors preventing or retarding aggregation prevent the spectral effect appearing; thus on going from pH 9 to pH 10, $\Delta\varepsilon_{252}$ is reduced on inactivation, and vanishes above pH 10. This effect can be related to the depolymerization (indicated by the scattering), which involves longitudinal splitting in the myosin molecule [8], which begins at pH 9 and finishes at pH 10. In alkali denaturation the effect only appears after neutralizing the solution, which allows of aggregation. No effect is found in guanidine solutions, in spite of the inactivation. Hence myosin can be irreversibly inactivated and denatured without an increase in true absorption if depolymerization can occur.

The point where the rise is irreversible (pH 11) coincides with that where the viscosity of actomyosin rises sharply, or the birefringence falls [9]; here alkali denaturation, distinct from inactivation, occurs. Hence the rise in true absorption is related to post-denaturation aggregation, which is produced by heating. This effect is completely suppressed by ATP at levels much lower than are required to protect myosin from thermal inactivation, or to reduce the birefringence of actomyosin. The ATP probably only suppresses the spectral changes by preventing aggregation, which occurs after inactivation and particle shortening, i.e. after intramolecular changes in which ATP is more profoundly and specifically involved.

Cysteine does not prevent depolymerization at the levels used, but merely makes the protein behave as though freshly prepared. The ageing (which is accompanied by aggregation [10]) may be caused by oxidation.

SUMMARY

(1) The absorption of myosin solutions in the ultra-violet has been measured, and the true absorption and scattering at 252 and 276 mμ have been calculated. The true absorption values for the active and inactivated protein are compared. Temperature (37°C), acid (pH 5·0), alkali (pH 11·0, followed by neutralization) and $CdCl_2$ increased the true absorption at 252 mμ by 23–47 per cent, and at 276 mμ by 5–14 per cent. The effect can be related to a single type of structure change, which does not occur at $pH \geqslant 10{\cdot}0$. Heat and acid increased the light-scattering, but $CdCl_2$ and alkali did not.

(2) On adding alkali to $pH \leqslant 10{\cdot}5$ we got only a reversible increase in absorption, and the above effect was not found. The alkali denaturation point should be taken as pH 11, while pH 10 corresponds to alkali inactivation. The latter is thus to be distinguished from the former as a more profound structural change causing no spectral change.

(3) Heat inactivation considerably preceded any spectral changes. Guanidine together with heat only increased the apparent absorption.

(4) The rises in true absorption and scattering on heating were completely suppressed by ATP if present as 12·5 molecules per molecule of protein; it did not prevent alkali denaturation.

(5) The spectral changes were probably related to post-denaturation aggregation. A possible structural mechanism is discussed.

Translated by J. E. S. BRADLEY

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THE RELATION OF THE SORPTION OF A VITAL DYE BY THE SPINAL GANGLIA OF THE FROG AND THE FUNCTIONAL STATE OF THESE TISSUES*

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Experimental material which has accumulated recently shows the common origin of local and spreading excitation. Substantial changes during local excitation have been studied in adequate detail. In the work of Nasonov and his co-workers attempts were also made by the method of vital staining to detect substantial changes in the conducting tissues during the spread of a wave of excitation.

Changes in the sorption of vital dyes during excitation in the nerve conductor have been found in non-myelinated nerves of invertebrates [1, 2] and in the myelinated nerves of the frog [3]. Changes in the sorption of the nerve cells during stimulation of the nerve have been demonstrated by workers investigating the spinal ganglia of the frog [4, 5], and also the spinal and cervical sympathetic ganglia of rabbits and cats [6–9].

It has been shown by quantitative methods that during effective excitaion the sorption of vital dyes by nerve fibres and nerve cells increases. In the work mentioned above it was found that changes in sorption were not observed always, and in order to obtain clear results certain conditions were essential (functional condition, strength, frequency and duration of stimulation). However, no specialized investigations of the conditions in which definite changes in the sorptive properties take place have been carried out.

In the present work an attempt has been made to relate the characteristic features of the changes in the tissues during stimulation to their functional condition.

METHODS

The experimental subjects were sciatic nerve preparations of the frog (*Rana temporaria*) with the spinal ganglia and roots.† The experiments were performed in March and October to November 1956. The sciatic nerves of the frogs were dissected out bilaterally, starting at the knee joint, and after opening the vertebral canal, the

* Biofizika **3**: No. 4, 413–421, 1958.

† The nerve cells of the spinal ganglia probably do not take part in the process of excitation directly. This is suggested by the experiments of Bethe [10] and Steinach [11], who showed the possibility of conduction with exclusion of the nerve cells. Nevertheless in the cells of the spinal ganglia a wave of excitation does arise, when the impulse passes along the corresponding nerve fibres. This was clearly demonstrated by the investigations of Svaetichin [12], who used micro-electrodes and succeeded in detecting the presence of an electrical wave of stimulation arising in the body of the ganglion cell during stimulation of a peripheral nerve.

eighth and ninth pairs of spinal ganglia were extracted. Removal of connective tissue fragments and calcified material and testing of the anatomical intactness of the preparation was carried out under a binocular loupe, the preparations being kept throughout in Ringer's solution. After cleaning, the preparations were kept in this solution for 1·5–2 hr, and then transferred to a humid chamber. The construction of the chamber allowed an experiment to be performed simultaneously on four pairs of preparations.

The order of the experiments was as follows: the distal end of the nerve was placed on stimulating silver electrodes with an interpolar distance of 4 mm, the central portion of the nerve was in contact with an earthed silver plate and the proximal part of the nerve, for about 2–2·5 mm below the ninth spinal ganglion, was placed on the sampling electrodes, connected to the input of a symmetrical amplifier of an oscillographic apparatus. With this arrangement of the apparatus the ganglia hung freely from the sampling electrodes. As a stimulus we used rectangular impulses of negative polarity, of 0·08 msec duration and frequency 100c/s.

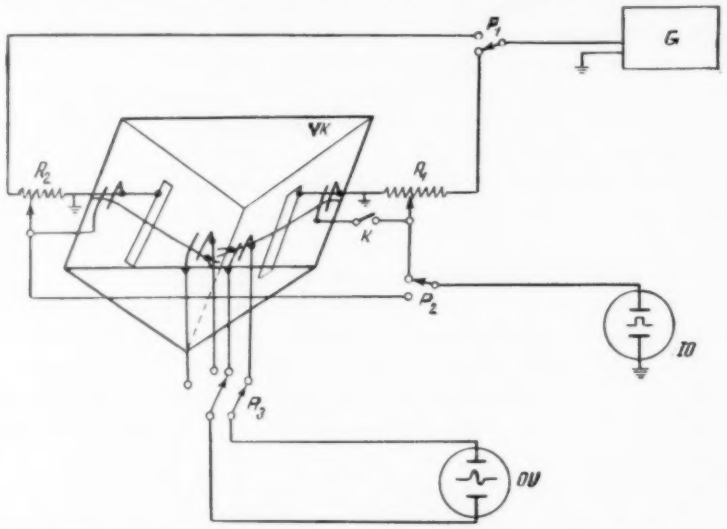

Fig. 1. Diagram of the apparatus. VK—humid chamber; G—generator of rectangular impulses; IO—graduated oscillograph; OU—oscillographic apparatus for registration of the electric potential of the nerve; R_1—potentiometer for testing and establishment of the value of stimulation of the experimental preparations; R_2—potentiometer for testing control preparations; P_1, P_2, P_3—switches; K—switch for the stimulation circuit. For simplicity the diagram of stimulation and testing is shown on one pair of preparations only.

The amplitude of these impulses was estimated by potentiometers (R_1 in Fig. 1) and was determined by the graduated oscillograph in conventional units proportional to the amplitude reading of the tension of the stimuli.

For all the preparations the threshold strength of excitation was determined. The threshold was established before the appearance of the first signs of a current peak. Next the strength of the stimulation was gradually increased to a level when its further increase caused no increase in the amplitude of the current peak. This

magnitude of the tension of the stimulation impulses, determining the threshold for the least conducting fibres of the nerve and exceeding that for the more highly conducting fibres by 2·18 times on the average, we will in future call the threshold of maximum, for simplicity.

Stimulation of four experimental preparations was carried out by a tension equal to the threshold of maximum, corresponding to the magnitude established by preliminary measurement for each preparation. Stimulation began after filling the lower part of the chamber, where the proximal ends of the preparations were to be found, with a solution of vital dye, and was continued for 40 min. The control pairs of preparations were in the same solution but were not subjected to stimulation. After stimulation the dye was poured off and measurement of the threshold again carried out. Next the preparations were taken out of the chamber, rinsed in Ringer's solution, and, under the binocular loupe the ganglia were separated from the nerve and roots and placed in tubes with 0·6 cm^3 of 70% alcohol, acidified with sulphuric acid. In the tube were placed two ganglia from the control or experimental preparation. Extracts were estimated colorimetrically in a Pulfrich photometer. The quantity of combined dye was shown by the value of the extinction coefficient E (optical density of the solution), proportional to the concentration of dye. The ratio of the value of the extinction in the experiment to that in the control, taken as 100 per cent, showed the change in the sorptive power of the tissues of the ganglia as a result of stimulation.

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RESULTS AND DISCUSSION

Six main series of experiments were carried out. In five series a 0·1 per cent solution of neutral red, prepared in Ringer's solution without sodium bicarbonate was used as a dye, and in one series a 0·05 per cent solution of phenol red.

Each series was performed on a separate group of frogs and was made up of investigations on 7–12 pairs of preparations. The results of the observations are shown in Table 1. Let us first consider the experiments in which staining was by neutral red. As can be seen from Table 1 (column 5), in three series (Nos. 1, 2 and 4) it was found that stimulation of the nerve causes a considerable and statistically significant reduction in sorption. In series No. 3 increase in the sorption was obtained, and in series No. 5 stimulation caused no change in the staining of the ganglia. Thus the changes in sorption due to stimulation differ considerably in the various series, and sometimes (series Nos. 2 and 3) they are directly opposite.

In experiments on the spinal ganglia of the frog, weakening of sorption of vital dyes during stimulation has never been observed before, which compelled us to pay particular attention to the results we had obtained.

Microscopical examination of the ganglia which had been immersed for 40 min in a 0·1 per cent solution of neutral red showed that granules of dye could be seen quite clearly in the nerve cells, in contrast to a background of diffusely stained protoplasm. Apart from differences in the intensity of staining which were often observed, the microscopic picture in the experimental and control preparations appeared identical.

In case of the use of vital dyes which form granules, diminished sorption of the dye may be found as a result of suppression of granule formation. In order to ascertain whether this occurred during our experiments, we performed experiments

Table 1. Mean values of the sorption of dye by the spinal ganglia and changes in their thresholds during stimulation

Month	Series No.	Sorption of dye				Changes in thresholds during stimulation in experiment compared with those in control ($P\% - 100\%$)
		Dye	Number of experiments (n)	Percentage changes in sorption during stimulation $\left(\frac{E_0}{E_k} \times 100\right) - 100$	Extinction of control ganglia (E_k)	
1	2	3	4	5	6	7
III	1	0·1% neutral red	7	−29·6±5·9	0·540±0·10	+55·6±23·6
X	2	0·1% neutral red	12	−20·4±4·3	0·389±0·03	+34·4±5·7
X	3	0·1% neutral red	10	+26·6±11·1	0·264±0·02	+22·2±11·6
XI	4	0·1% neutral red	12	−11·8±3·6	0·670±0·04*	+43·0±5·7
XII	5	0·1% neutral red	12	−0·2±8·6	0·375±0·03	+29·8±9·3
XII	6	0·05% phenol red	2 (24)	−22·4	(0·017)	+31·4±6·2

* For technical reasons the absolute values of the extinction of the control preparations in series No. 4 were determined at a value much too high, and cannot be compared with the results obtained in the other series.

with phenol red (a dye which does not form granules). In view of the fact that phenol red stains tissues more feebly, the tubes with alcohol each contained only 6 pairs of ganglia. Investigation of 12 pairs of preparations showed that sorption during stimulation was diminished compared with the control by 22 per cent. Hence it follows that this diminution cannot be attributed to suppression of granule formation.

The diminished sorption which we obtained cannot be explained by the fact that in contrast to the above cited workers, we used slightly different stimuli and higher concentrations of dye. As proof of this there is firstly the fact that in other series of experiments under exactly similar conditions contrary results were obtained, i.e. stimulation caused an increase in sorption. Finally, in a special series of experiments in which as stimulation we used an induction coil, and the concentration of neutral dye was reduced to 0·005 per cent, stimulation also caused a diminution of sorption (mean value, $M = -16 \pm 6{\cdot}6$ per cent).

Since in all the series of experiments the conditions were absolutely identical, it appeared that the difference in the results of the individual series (see Table 1) could be explained only by the special features of the condition of the preparations.

Seasonal changes did not play any part in our experiments, since different and even directly opposite results were obtained in the course of the same month, although the groups of frogs were kept under different conditions. The frogs in all cases were kept in running tap-water. However, in the series in which stimulation caused increase of sorption, the frogs were taken from an aquarium in a room at a temperature of about 0°C, and in the series in which there was diminished sorption, from an aquarium in a room at room temperature.

In order to establish the causes of the different changes in sorption as a result of stimulation, this one fact was not sufficient, and therefore it was essential to find a means of estimating quantitatively the changes in the condition of the preparations.

The investigations of Nasonov and his co-workers showed that the functional condition of the tissues can be determined by their ability to combine with a vital dye. Accordingly in defining the initial state we attempted to use the value of the extinction of control ganglia (E_k), determining the sorptive power of preparations which were not subjected to stimulation. From Table 1 (column 6) it can be seen that the mean values of extinction of the control preparations are different in the different series. If we compare the value of E_k with the values of the changes in sorptive power as a result of stimulation (column 5), it is easy to see that with high values of extinction of the control stimulation diminishes the sorptive power of the ganglion tissues, but at relatively low values of extinction of the control an increase in sorption is observed during stimulation. This relationship is shown in Fig. 2(*a*).

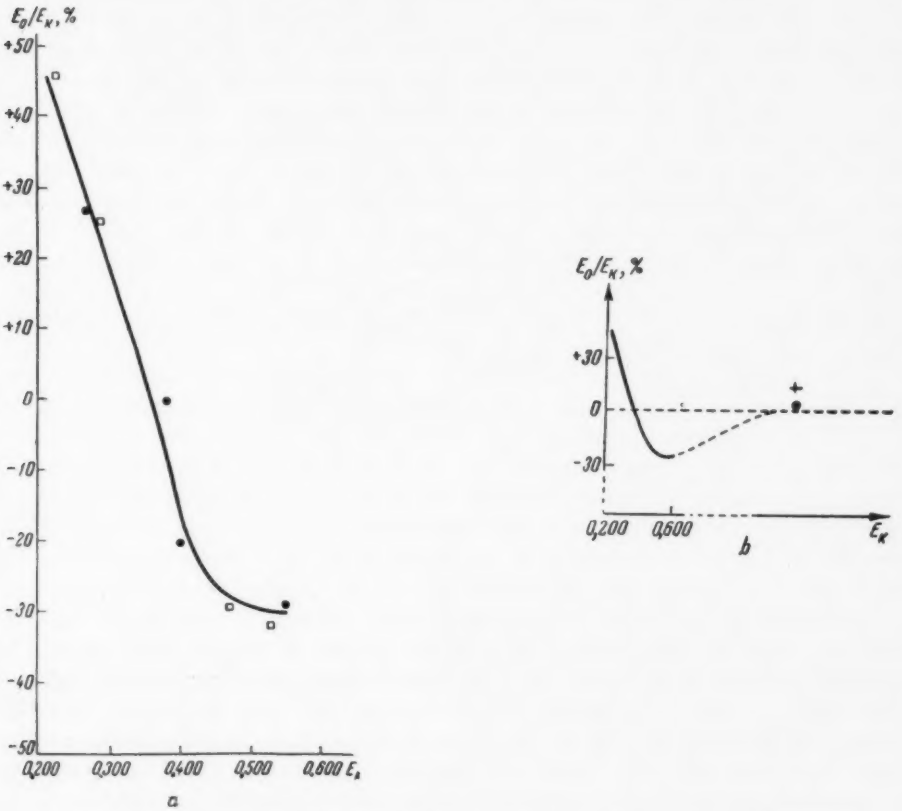

FIG. 2. (a) Graph of sorption of dye by the spinal ganglia of the frog during stimulation vs. the value of extinction of the control preparations. Abscissa: values of extinction of the control preparations (E). Ordinate: changes in sorption of neutral red as a result of stimulation, $\left\{\left(\frac{E_0}{E_k}\times 100\right)-100\%\right\}$. The dots represent points corresponding to the mean values for the separate series of experiments; the squares are points obtained by selective treatment of the results of all the series (for details see the description on p. 402). (b) The dotted line indicates the supposed course of the curve with further deterioration of the condition of the preparations, as far as death.

The curve indicates the relation of the change in sorptive power to the functional state of the preparation, as shown by extinction of the control.

A similar relationship was found also in isolated series of experiments. If, for example, the peculiar scatter of the results of the experiments in series No. 2 (Fig. 3) is examined, a definite tendency of the scatter can be recognized which is in the direction of the curve shown in Fig. 2(*a*). This suggests the existence of a variable systematic error in the measurements in the whole series, which is also the result of the relationship of the changes in sorption to the initial condition.

One further confirmation of the existence of the relationship described above can be quoted. If, proceeding from the curve in Fig. 2(*a*), we determine the value of extinction of the control at a stimulus applied by us which causes no change in sorption ($E_k(0)=0{\cdot}370$), and then from the total number of experiments which are not related to this series we select those in which the value of E_k would exceed $E_k(0)$ by 25 per cent or more, and then those where E_k would be at least 25 per cent less than $E_k(0)$, it will be found that in the first group there are almost exclusively experiments in which stimulation caused diminished sorption (mean value of the change in this group, $M=-29\pm6{\cdot}2$), and in the second group, experiments in which increase in sorption was mainly observed ($M=+25{\cdot}2\pm10{\cdot}7$). If we select in the first group cases where E_k is over 40 per cent higher than $E_k(0)$, and in the second group cases where E_k is over 40 per cent lower than $E_k(0)$, the mean values of the change in sorption calculated for these groups will be greatly increased in absolute terms (in the first group $M=-32\pm3{\cdot}4$, in the second $M=+45\pm13{\cdot}2$), and their statistical significance is also increased. The points obtained by a selective treatment of this sort are shown by squares in Fig. 2(*a*).

Consequently, at a certain functional state of the preparations, characterized by low values of sorption in the control preparations, the stimulation which we applied as a rule caused increase in sorption of vital dyes and, on the other hand, at high values of extinction of the control preparations stimulation regularly caused diminished sorption. We may also observe that there is a certain condition in which stimulation does not cause any noticeable change in the sorptive power. Thus, for example, in series No. 5, where the mean value of the extinction of the control was $0{\cdot}375\pm0{\cdot}027$, the change in sorption during stimulation did not occur ($M=-0{\cdot}2\pm3{\cdot}6$).

It may also be supposed that an index such as the sorptive power (E_k) must be related to other characteristics of the functional state of the preparations, and in particular to the level of excitability of the nerve trunk. However it was found that the absolute values of the threshold levels determined during rhythmic stimulation were almost identical in all the series. Even in those series (Nos. 2 and 3), in which sharp differences were observed in respect of the values of E_k and correspondingly different changes in the sorptive power during stimulation, the mean values of the thresholds varied by not more than 1 per cent. Thus in series No. 2 the mean value of the threshold was 39·7 mV and in series No. 3 it was 39·3 mV.

It is known that methods using functional indicators are found to be more sensitive, and may reveal relatively fine changes in the functional condition. In the experiments described, the stimulation to which the preparations were subjected (stimulation by a reactangular pulse of duration 0·08 msec, and repetition frequency 100 c/s, acting for a period of 40 min), may be regarded as some form of functional

load, unchanged throughout all the series of experiments. As the observations showed, the thresholds after stimulation were as a rule different from their original values. The value of the change in the threshold in the experimental preparations may be defined by the ratio of the value of the threshold after stimulation to its original value: P_0=threshold after stimulation/threshold before stimulation.

It must be mentioned that leaving the preparation itself in a humid chamber for 40 min caused some change in the value of the thresholds, as may be seen from comparing the value of the thresholds of the control preparations at the beginning and end of the experiment: P_k=threshold after 40 min stay in the chamber/threshold at the beginning of the experiment.

The ratio $P_0/P_k=P$, expressed in per cent, is a value which shows the degree of change arising in a nerve as a result of electrical stimulation compared with a control, i.e. the value is characteristic of the power of the nerve to maintain this particular functional load.

The value P (column 7) differs greatly in the various series of experiments. The curve shown in Fig. 4 is constructed in a similar way to that in Fig. 2, the only

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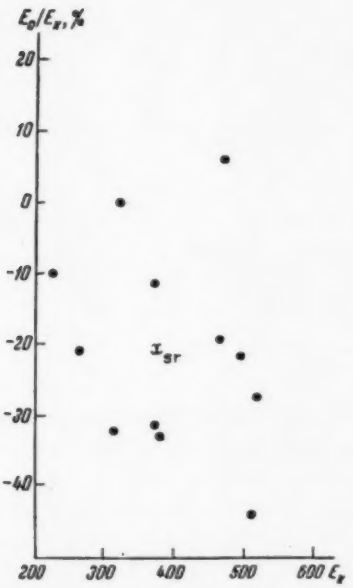

FIG. 3. Scatter of experimental points in series No. 2. The units are the same as in Fig. 2. The points give the results of the individual experiments; x_{sr} is the mean value for the series.

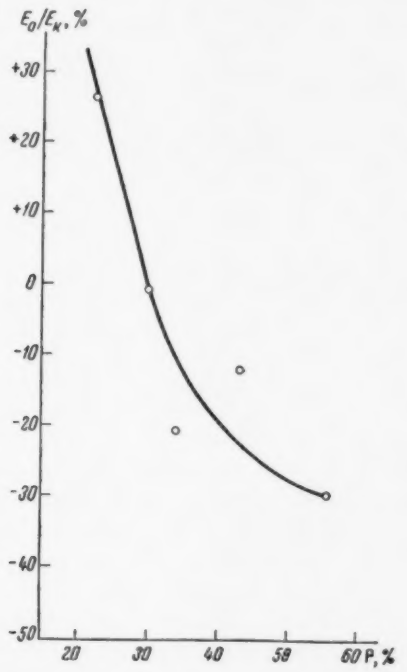

FIG. 4. Graph of sorption of neutral red vs. changes in the thresholds during stimulation. Abscissa: values of the threshold P in per cent. Ordinate: changes in sorption as a result of stimulation, $\left\{\left(\frac{E_0}{E_k}\times 100\right)-100\%\right\}$. The points on the curve show the mean values for the separate series of experiments.

difference being that along the axis of the abscissa in place of extinction of the control preparations are shown the values P, expressed in per cent. The relationship shown in the graph illustrates that with considerable changes in the thresholds (low resistance of the preparations to stimulation) the sorptive power of the tissues of the ganglia is diminished in comparison with the control, and on the other hand, with slight changes in the thresholds (high resistance of the preparations) sorption is increased as a result of stimulation.

The relation of the changes in sorption during stimulation to the functional state is essentially unaltered by replacement of P by E_k (see Figs. 2 and 4), i.e. these two indices of the functional state are connected with each other by a linear relationship. In fact, if a graph is constructed (Fig. 5) in which the abscissa is P and the ordinate is E_k, and we insert the mean values of these factors in the different series of experiments, it will be found that the experimental points will be situated close to a line expressing a direct proportionality between these factors. Hence it follows that the functional characteristics of nerve cells and axons are changed to the same extent within the limits of our observations. For this reason, by the state of the nerve trunk we may indirectly judge the state of the cells.

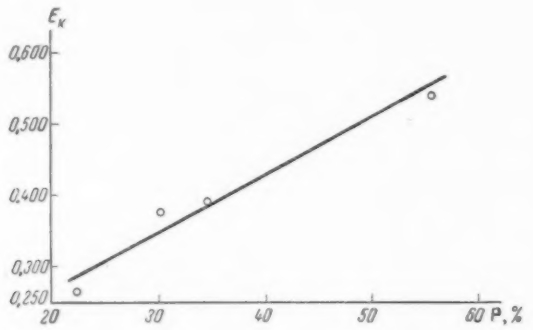

Fig. 5. Relationship between values of E_k and P. Points on the curve are mean values for separate series of experiments.

The results of the experiments permit the conclusion that the direction and degree of change in the sorptive power of the spinal ganglia during effective stimulation depend on their original functional condition. It may be thought, that in the frog kept in a warm atmosphere during the winter and early spring, deleterious changes may take place in the state of the tissues which would be revealed (in particular, in increased sorption of dye by the control preparations (increased E_k) and by diminished resistance of the nerve conductor to the action of the electric current (increased P)) and that stimulation would cause a diminution in sorption of vital dye by the tissues of the spinal ganglia. The relationship which we found between changes in sorption during stimulation and the initial condition accounts for the difference in the results which took place in the different series of our experiments. It is possible that a similar explanation accounts for the discrepancy between our results and those of other workers who did not observe diminished sorption of dye by spinal ganglia in the frog during stimulation of the nerves.

Diminution of the sorptive power of the spinal ganglia during stimulation was observed earlier only in experiments on warm-blooded animals. Romanov [7] observed diminished sorption by the ganglia of the rabbit at a definite phase of the after-effect of stimulation (after 40–80 min). Diminished sorption of vital dye was found by Zarakovskii and Levin [9] during sub-threshold stimulation of nerves, while during super-threshold stimulation only increase of sorption was observed. The above-mentioned authors explained the diminution of the sorptive power by the fact that weak stimulation or the after effects of stimulation increase the resistance of the preparations to a traumatizing influence—in this case to the toxic action of the dye.

In our experiments diminished sorption arose during the action of a super-threshold stimulation, and if we accept the above quoted explanation we shall have to admit that in a relatively poor functional state an increase in resistance may be caused by stimulation far in excess of the threshold. In this case, on the basis of the relationship shown in Figs. 2 and 4, the conclusion might have been drawn that the resistance of the preparation increases as the functional state worsens. The results given above, however, confirm that this is so only for a definite range of changes in the functional state, corresponding to those values of E_k and P which are indicated on the abscissae. Changes in sorption during stimulation when the functional condition is better than that corresponding to values of $E_k \approx 0{\cdot}540$ and of $P = +55{\cdot}6$ per cent were not studied by us. For this reason the further course of this relationship is still unknown. However there are sufficient grounds for supposing that this relationship, having an extreme in the negative region of values of E_0/E_k, must eventually tend towards zero, since in an extremely poor state of the preparation (death) stimulation cannot produce any change, i.e. the values of sorption of dye in both the experimental and control preparations will be equal (0 per cent on Fig. 2 *b*). Hence it follows that the same value of diminution of sorption during stimulation may be observed in at least two quite different functional states.

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SUMMARY

(1) During stimulation of the sciatic nerve with rectangular impulses of duration 0·08 msec and frequency 100 c/s it was found that stimulation may cause both increase of sorption of neutral red by the spinal ganglia, or its diminution.

(2) The direction of the changes in sorption and the magnitude of these changes depend on the initial functional state of the preparation.

(3) The initial functional state of the preparation was found to be definable in terms of the value of sorption of vital dye by the spinal ganglia of the control preparations, and also by the power of the nerve to carry its functional load, which can be judged by the change in the threshold as a result of stimulation.

(4) With a low value of sorption of the control and slight changes in the thresholds, stimulation increased the staining of the ganglia. With higher values of sorption of the controls and considerable changes in the thresholds of the nerve as a result of stimulation, the staining of the ganglia was weakened by the latter.

(5) There was a direct relationship between the sorptive power of the control ganglia and the changes in the thresholds of the nerve as a result of stimulation.

Translated by B. HAIGH

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RHYTHMIC BIOLECTRIC PHENOMENA IN THE SINGLE-CELLED ORGANISM *OPALINA RANARUM* *

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THE irritability, galvanotaxis and summation of stimuli in *Paramecium* have been studied in our laboratory recently. It has been shown that the physiological effects depend on the metabolic state, and in particular on the states of the sulphydryl groups and of the acetylcholine–cholinesterase system [1–4]. These studies have been extended to cover the relation of metabolism to bioelectric activity in other simple organisms. This type of study was begun by Kamada [5] and Kinosita [6], who described the membrane potentials of *Paramecium* and *Opalina*, and showed that these were related to the metachronic ciliary waves. Very recently Hisada [7] has described studies using glass microelectrodes on *Noctilucas scintillans*, and has demonstrated rhythmic action potentials related to division. Here we report some work in *Opalina ranarum*, an infusorian parasitic on frogs' intestines.

MATERIALS AND METHODS

Opalina ranarum cells are some 70–100μ × 150–200μ, and about 20μ thick. The cells were removed from a frog's intestine 1 hr prior to use and placed in a mixture of solutions of 0·125 M NaCl, 0·125 M KCl and 5·33 M $CaCl_2$ in the ratio 100: 2·5: 3. The intracellular potentials were picked up with glass microelectrodes of outside diameter 1–2μ, filled with 3 M KCl solution. An Ag/Ag Clelectrode was placed in this solution. The second electrode (Ag) was non-reactive and was placed in the solution around the cell.

The microelectrodes were inserted at an angle from above under the microscope, using a drop of suspension in a hollow in a slide. The cell was clamped between the

* Biozikfia **3:** No. 4, 422–425, 1958.

electrode and the slide. Fine bent glass tubes were used to produce a continuous flow of liquid through the drop, or to change the composition. Control experiments were done under the same condition, but without inserting the electrode into the cell. A d.c. amplifier with a pass band from 0 to 2000 c/s was used, with a special electrometer head of input resistance 200 MΩ and gain about 0·9. This head was fixed to a stand fitted with a micrometer screw, and served to hold the microelectrode. An EO-7 oscilloscope was used for visual studies, and a loop oscillograph for recording on film.

RESULTS

Inserting the electrode into the cell always caused a sharp, very rapid, and clearly visible step in the potential. A slow positive rise before contact was made was almost always seen. The step was always very stable, even for organisms taken from different animals, and varied from 25 to 45 mV.

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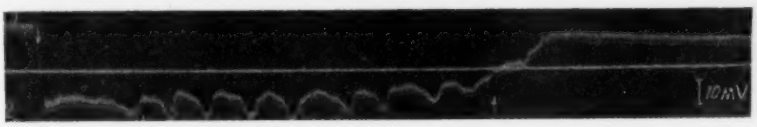
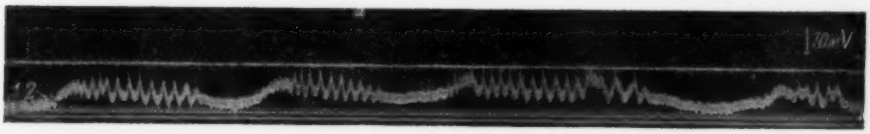


FIG. 1. Slow oscillations in the intracellular potential. (1) Intracellular potential, (2) time marks (1 sec). The arrows denote where the electrode was inserted or removed.

After the step, the periodic variations were seen; these gave the impression of passing through some mean line (Fig. 1). The repeat period varied widely (7–35 sec), particularly for organisms from different sources. The minima were usually flatter than the maxima, but not always. The amplitudes varied from 5 to 20 mV. These oscillations sometimes began immediately, or sometimes were delayed somewhat (up to a minute); if immediate they were more frequent and less regular in the first minute, then became regular throughout (the longest period of observation was 30 min). A reverse step occurred on removing the electrode at the end of the experiment (Fig. 1). The zero line produced by the readings before and after the experiment was used as the reference line.

More rapid oscillations could often be seen superimposed on the slower ones;



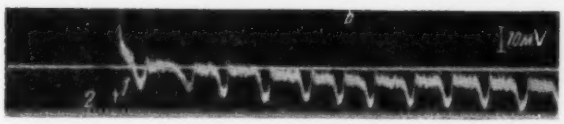


FIG. 2. Various forms of the oscillations. In (a) and (b) faster oscillations are seen on the minima in the slow oscillations. (1) Intracellular potential, (2) time marks (1 sec). The arrows denote where the electrode was inserted or removed.

these were of 2–3 sec period, and 1–6 mV in amplitude. These oscillations were also regular, and appeared on a definite section of the slow wave (usually the minimum).

The rate at which the solution flowed through the drop greatly influenced the rate of the slow waves. At very high rates (0·2 ml/sec) the frequencies of slow and fast waves became similar, and the waves almost fused. The ionic composition also influenced the effect. Potassium produced a rapid and prolonged reversion of the cilia [8, 9], and also influenced the oscillations. Isotonic KCl (M/8) causes the potential to fall to that of the solution; if the KCl was washed away the potential returned to normal. Lower KCl concentrations (M/90) did not affect the potential but greatly slowed down the oscillations; if the KCl was washed away the oscillations returned to normal (Fig. 3).

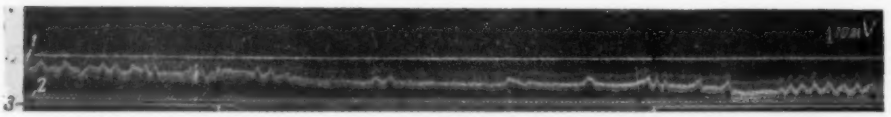


FIG. 3. Effect of KCl (M/90) on the oscillations. (1) Intracellular potential, (2) time marks (1 sec), (3) effect of KCl. Before and after the KCl was washed with the usual salt mixture.

The tests also showed that the oscillations were dependent on the osmolarity, the fast waves becoming faster in hypotonic solutions, while in hypertonic ones (produced by adding sucrose) they became slower. Our previous data on *Paramecium*, and Kinosita's [6] on the relation of resting potential to ciliary activity, led us to relate these oscillations to metabolic rates. Our experiments on this showed that adding 2:4-dinitrophenol (10^{-4} M) to prevent oxidative phosphorylation resulted in a gradual slowing-down and final cessation of the oscillations; the fast oscillations vanished before the slow. Washing with the mixed salt solution for 10–15 min was required to restore the oscillations, whereas ATP (8×10^{-7} M) restored them in 1–2 min. These data indicate that bioelectric rhthyms dependent on the medium and metabolic state occurring in single-celled organisms.

SUMMARY

Glass microelectrodes of outside diameter 1–2μ and oscillographic recording were used to record the intracellular potentials of *Opalina ranarum*; inserting the electrode caused a clearly visible step in the potential. Oscillations followed on the step; the period varied from 7 to 35 sec, and the amplitude from 5 to 20 mV. Fast oscillation of periods from a fraction of a second up to 2–3 sec, and of amplitudes 1–3 mV were superimposed. The period of the slow oscillations was greatly influenced by the flushing ratio, by the osmolarity, by K ions and by metabolic inhibitors (2:4-dinitrophenol).

Translated by J. E. S. BRADLEY

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THE EFFECT OF AN ULTRA-HIGH FREQUENCY FIELD ON THE FUNCTIONS OF THE NERVOUS SYSTEM*

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As our previously published paper indicated [1], the effect on the nervous system of physical agents used in modern technical science is a matter of great scientific interest from both theoretical and practical viewpoints. Those physical factors which have a deep action and directly affect various elements of the nervous system under certain conditions deserve special study; ultra-high frequency (u.h.f.) fields fall within this class. In spite of the fact that the fundamental experimental data on the action of u.h.f. on the nervous system were gathered in the 1930s and early 1940s, only isolated publications appeared after this period.

We thought it useful to publish this survey because research on this problem has not been published in full, and this research has retained its value up to the present through the widespread application of u.h.f. in medicine and the technical sciences.

A considerable amount of the research on the effect of a u.h.f. field on the nervous system was carried out at a time when u.h.f. dosimetry was little developed; when setting out data we will adhere to the u.h.f. dose classification used by the respective authors. The most usual method in early research on the action of u.h.f. on the body was to classify the doses according to their biological effect (lethal and sublethal doses) or according to the presence and rate of heating of the tissue, organ, or whole body, whichever is the case (pyretic, subpyretic, oligothermic, etc.). It is obvious that this dosimetry is unsatisfactory, but to express it in modern, generally accepted dosimetric units on the basis of the experimental conditions described in the papers would necessitate special research and would be impossible in many cases.

In view of the short space available, the characteristics of the u.h.f. field will be described only so far as is necessary to understand the research described.

GENERAL STUDY OF THE REACTIONS OF THE NERVOUS SYSTEM TO A U.H.F. FIELD

Many workers had become aware of the high sensitivity of the nervous system to u.h.f., even when these were not widely used. Experimenters using a u.h.f. field in experiments on animals observed that excitation occurred in animals in a u.h.f. field [2–5], and also that such a field produced drowsiness and sleep in animals of

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various species [6]. The functions of the nervous system in a person systematically exposed to relatively large doses of u.h.f. are impaired [7–18].

Highly effective methods of protecting personnel are used in modern radar and ultra-short-wave stations. In the opinions of some workers the ultra-high frequency field is no longer an important source of industrial injuries among the personnel servicing these stations. However, it may have a harmful effect in other working conditions, where there is a possibility that a u.h.f. field may be formed, either periodically or permanently, due to ineffective screening of apparatus [19, 20].

THE EFFECT OF A U.H.F. FIELD ON THE FUNCTIONS OF THE CORTEX OF THE LARGER CEREBRAL HEMISPHERES

Exposure to u.h.f. fields of high intensity accompanied by a considerable increase in body temperature causes drastic inhibition of the functions of the cortex. Heller [21] exposed hens' heads to a u.h.f. field of high intensity; as a result of this symptoms of complete paralysis were observed. The hens did not eat, drink or change their position of their own accord. When put in a standing position they retained it until they collapsed from exhaustion. Autopsy revealed hyperaemia and a reduction in the size of the cerebral hemispheres. The paper contains no information on the result of histological examination.

Lebedinskii [22] observed that the overall exposure of persons suffering from progressive paralysis to pyretic doses of u.h.f. for purposes of treatment produced temporary inhibition of the functions of the cortex, taking the form of a deterioration in control, intensification of the pathological reflexes and weakening of the mental functions.

Kharchenko [23] showed that exposing a pigeon's head to a u.h.f. field produced by a 35 W generator for a period of 3–5 min caused the latent period in the conditioned motor feeding reflexes to extend to twice its normal length. After numerous exposures to a u.h.f. field produced by a 300 W generator, the positive conditioned reflexes disappeared, and were only slowly renewed.

Glezer [24] studied changes in conditioned reflexes due to exposing dogs' heads to a u.h.f. field. He showed that exposures which caused the animal no discomfort stimulated both conditioned and unconditioned reflexes. Inhibition processes in the cortex of the greater hemispheres were reduced after repeated exposures. Differentiation disappeared almost completely.

The initial functional state of the cortex and the typological features of the animal play an important part in the reactions of the cortex to u.h.f. Promtova [25] found that repeated exposure to u.h.f. has varying effects on dogs in which higher nervous activity is either normal or impaired (the dog's head was exposed for 10 min, with wavelength 7·7 m power in the circuit 90–100 W, electrode diameter 10 cm, clearance 5–6 cm). Some of the conditioned secretory feeding reflexes in two dogs with a normal conditioned reflex background were stimulated in the first phase, becoming stable at the upper limit of their initial state while preserving differentiation; in the second phase the conditioned reflexes were drastically retarded, and differentiation diminished. Normal functioning was resumed 10 days after the conclusion of the exposure. Where the dogs' higher nervous activity was impaired as a result of previous suppuration at the basal region of the cranium, the first phase of a similar exposure

produced a deterioration in the pathological state of the cortex; the second phase brought the animal's higher nervous activity to a normal state, which was retained for two months after the conclusion of exposure.

Variations in the same animal's reactions to the effect of a u.h.f. field indicate that the effects of u.h.f. are dependent upon the functional state of the cortex [26]. In some experiments relatively localized exposure of the temporal and frontal regions of a dog's cortex by means of the contact electrodes designed by Terent'eva [27] at a frequency of 50 Mc/s (intensity of field to which the subject was exposed, 7–55 W; active electrode diameter 25 mm), produced distinct changes in the conditioned feeding secretory reflexes when the u.h.f. field strength was 7–12 W, whereas in other experiments exposure to a field of strength 20–55 W produced no effect.

The reactions of the cortex to u.h.f. are dependent upon the type of higher nervous activity which the animal has. Bilateral treatment of the auditory regions by the above-mentioned method produced a reversible reduction in the conditioned reflexes, or inhibition of them, within the limits of the auditory analyser only in three dogs with strong higher nervous activity. Both bilateral and unilateral treatment of the auditory region reduced the conditioned reflexes from various analysers in a dog with weak higher nervous activity [26].

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The reactions of the cortex to exposure to u.h.f. may also be found by the electrophysiological method [28]. An electro-encephalograph from rabbits exposed to u.h.f. shows an increase in wave frequency and amplitude (the physical characteristics of the u.h.f. field used in this work are not given).

Reversible changes in some analyser functions occurred when the cortical areas of analysers in human beings were exposed to small therapeutic doses of u.h.f. Local u.h.f. treatment of the auditory region of the cortex produced changes in the thresholds of audibility in 26·6 per cent of the cases; in some experiments the thresholds were extended, and in others they were reduced [29]. Treatment of the sub-parietal lobe region produced short-term changes in spatial sound perception without threshold changes in 27 per cent of the cases [30].

Treatment by u.h.f. of the occipital regions produced changes in the course of consecutive visual images [31]. The action of a high-frequency field on the occipital region produces a reversible increase in the area of light-sensitivity, simultaneously reducing the area of colour-sensitivity [32].

No changes in the critical frequency at which instantaneous images merge were found to occur as a result of the bitemporal application of a u.h.f. field. However, the critical frequency increased somewhat when the u.h.f. field acted directly on the eyes [33].

Turlygin's work must be mentioned when considering research into the action of a u.h.f. field on the brain [34]; this work is of interest although he used centimetre waves which are outside the u.h.f. range. He exposed the heads of patients completely adapted to darkness to a diffused electric field and observed a supplementary increase in the sensitivity of marginal vision reaching 100 per cent.

Hoff and Weissenberg [35] studied motor reactions resulting from the action of a u.h.f. field on the cortex of the greater hemispheres in healthy individuals. During the experiment the subjects sat with their eyes closed and their hands extended in front of them. The unilateral application of a u.h.f. field to the frontal lobe of the cortex caused involuntary movement of the hands towards the active electrode.

THE EFFECT OF A U.H.F. FIELD ON THE FUNCTIONS OF THE CEREBELLUM

Hoff and Weissenberg [35] also studied motor reactions resulting from the action of a u.h.f. field on the cerebellum. The unilateral application of a u.h.f. field to a hemisphere of the cerebellum in the biventricular lobe region produced an involuntary movement of the hands towards the side opposite to that of the hemisphere exposed to the u.h.f. field. When the cerebellum region in dogs was exposed to a u.h.f. field, changes in conditioned reflex activity occurred; these changes were determined by the type of higher nervous activity in the animal and by the functional state of the cortex. In one of the dogs there was complete parallelism between the reaction of the cortex to the action of a u.h.f. field on the cerebellum and its reaction to bromide and caffeine.

Exposure of the cerebellum to a u.h.f. field in cases where the exposure had an effect both in small oligothermic doses and in large thermic doses affected conditioned reflex activity, reduced the conditioned reflexes and retarded the differentiation in three dogs with strong higher nervous activity. A course of exposure of the cerebellum to a u.h.f. field had a normalizing effect on two weak type dogs with unstabilized conditioned reflexes [36].

Treating the cerebellum region of human beings with therapeutic oligothermic doses of u.h.f. produced short-term changes in some of the visual functions, in light thresholds in darkness measured from the edge of the retina [37] and in the qualitative and quantitative characteristics of consecutive visual sensations [31].

THE EFFECT OF A U.H.F. FIELD ON THE FUNCTIONS OF THE INTERMEDIATE BRAIN

Bekauri [38] subjected a frog's head from which the olfactory lobes and cerebral hemispheres had been removed to local u.h.f. treatment and studied the Sechenov inhibition phenomena. This effect did not occur in frogs in which the connective cords had been severed. Reflex times did not increase when frogs' heads from which the visual protuberances and visual lobes had been removed were exposed to a u.h.f. field.

Sych [39] also studied suppression of the skin–acid reflexes in frogs as a result of exposure to u.h.f. Unilateral severing of the connective cords produced an increase in reflex times only on the side on which sympathetic innervation was preserved. References to works dealing with changes in the functions of the autonomic organs as a result of exposure to a u.h.f. field are set out in the earlier article [1].

THE EFFECT OF A U.H.F. FIELD ON THE FUNCTIONS OF THE SPINAL CORD

Exposure to large doses of u.h.f. accompanied by rapid heating of the tissues completely suppresses the functions of the spinal cord [21]. The action of a u.h.f. field on the latent period and the threshold of excitation in spinal reflexes has been studied in the greatest detail [40–45]. Some workers have come to the conclusion that small doses of u.h.f. shorten the latent period in the spinal reflexes in frogs, whereas large doses lengthen it [42, 44, 45].

In some experiments increases and reductions in reflex excitation occurred at various stages after one exposure to u.h.f. [42, 43].

Variations in the latent period and the threshold of excitation in the spinal reflexes occurred when the frog's spine was exposed to both overall and local u.h.f. treatment,

and the hind-limbs were outside the u.h.f. field; consequently, these phenomena are connected with changes in excitation in the central part of the reflex arc [42, 45].

Local u.h.f. treatment of various areas produces changes in the latent period and in some other characteristics of the knee-jerk reflex in warm-blooded animals. Moskaliuk [46] studied this phenomenon in detail, and showed that these changes are highly sensitive indicators of u.h.f. action. The latent period in the knee-jerk reflex was shortened as a result of doses many times smaller than the usual therapeutic doses.

THE EFFECT OF A U.H.F. FIELD ON THE FUNCTIONS OF THE MOTOR NERVES

Exposure to a u.h.f. field causes changes in excitation in the peripheral nerves. Audiat [47] found that exposing a myoneural preparation to a u.h.f. field reduces the nerve's rheobase. The rheobase increased rapidly after the u.h.f. generator was switched off, but if the temperature of the Ringer's solution in which the preparation was submerged was kept constant there was no increase in the rheobase. The method used in these experiments has provoked some criticism. Reznikova [48] pointed out that assessment of the rheobase during exposure to u.h.f. may be inaccurate, due to the effect of induced currents on the instruments used to measure excitation.

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Many workers have observed changes in excitation in a myoneural preparation in a u.h.f. field under conditions which are open to no objection (for example, when excitation is measured after the u.h.f. field has been switched off, or when the results are obtained from a part of the nerve outside the u.h.f. field, etc.). There is now no reason to doubt the possibility that a u.h.f. field affects the functions of the peripheral nerves, but one should take into account possible errors in measuring excitation in a myoneural preparation in a u.h.f. field.

Much of the work which will be examined below is open to these objections. However, we think that it would be wrong to reject out of hand all the work in which this method has been used. In many cases the results of experiments in which stimuli to determine excitation were applied while the u.h.f. field was still active coincide with those obtained when excitation was determined after the field had been switched off, or when the stimuli were applied to a part of the nerve outside the range of the u.h.f. For this reason we think that it will be useful to examine all the available material.

The problem of liminal doses of u.h.f. causing changes in the excitation of a myoneural preparation remains a debatable one.

When exposing a myoneural preparation to a u.h.f. field of total intensity 1–2 W producing no perceptible increase in the temperature of the preparation, Dalton [49] observed an increase in the latent period of muscular contraction and a reduction in the amplitude and duration of a single contraction when stimulation was indirect.

Hill and Taylor [50, 51] take the view that excitation of a myoneural preparation in a u.h.f. field can only be reduced by exposure to an intensity sufficient to heat the muscle to 42°.

In Khazen's experiments [52] excitation of a myoneural preparation changes as a result of exposure to a dose of u.h.f. which produced a 3–4° increase in the preparation's temperature in 10 min; however, the effect sometimes occurred immediately after the generator was switched on, i.e. before there could be any heating effect.

In the work described above, the myoneural preparation as a whole was exposed to a u.h.f. field. Therefore the functional changes observed may have been due to the action of the u.h.f. on the nerves, the myoneural endings and on the muscle itself. Published information indicates that all these factors do in fact have some effect.

Rubin and Vorob'ev [53] found that a u.h.f. field exercises a suppressive action on direct excitation of a calf muscle isolated from a frog. In this instance the muscle temperature increased by 1·4–7·6°.

Reznikova's work [48] established that excitation of a sartorius muscle isolated from a frog undergoes changes as a result of exposure to an intense u.h.f. field. In this process the rheobase increases but chronaxy is curtailed. In some cases normality was not even resumed after 5–6 hr. Excitation was studied in these experiments after the u.h.f. field had been switched off.

Reznikova exposed the whole muscle, i.e. the nerveless part and the part supplied with nerve fibres, to a u.h.f. field, so action of the u.h.f. field on the nervous elements of the muscle was not completely excluded. Mogendovich's work [54] provides information for the further analysis of these phenomena, showing that when a u.h.f. field acts on a muscle the extent of the muscular contractions is altered when stimulation is given by way of the nerve, when stimulation is direct, and after the myoneural apparatus has been excluded by curarization. The stimulating electrodes were outside the condenser field in all the experiments carried out by Mogendovich.

Alekseenko [55] observed that the action of a u.h.f. field of strength 3·5–7 W, wavelength 6·2 m, acting for a period of 5–10 min, produces diverse changes in rheobase and chronaxy in calf and sartorius muscles isolated from a frog. This effect was retained after curarization.

Lyudkovskaia and Alekseenko [56] used the same exposures in experiments on a sartorius muscle isolated from a frog, and observed an overall increase in the intensity of diffraction spectra of all orders in some cases, and an overall reduction in these spectra in others. This phenomenon was observed both in the nerveless part of the muscle and in the part supplied with nerves; it was not affected by curarization.

Various methods have been used to study the effect of a u.h.f. field on a nerve trunk. Grigor'eva's extensive and carefully executed research [57] showed that local 10 min exposures to a u.h.f. field stimulates the regeneration of the partially severed sciatic nerve in a cat, whereas 30 min exposures suppress the regenerative processes (conditions of exposure: plate area 10×15 cm, distance between the plates 20 cm, current in the measuring circuit 0·4 A).

Bekauri [58] found that the resting potential was reduced when the whole of the nerve was exposed to a u.h.f. Local u.h.f. treatment of an undamaged nerve produces an electro-negative response in the part of the nerve exposed to the u.h.f.

Reizin and Motsnyi [59] showed that functional changes produced by a u.h.f. field reach a maximum in that part of the nerve which lies between the condenser plates. The changes in excitation which were observed in remote parts of the nerve were only due to leakage from the field.

Mogendovich [60] showed that conductivity of both single and tetanic excitation waves was impaired in that part of the nerve which was exposed to local u.h.f. treatment. A few minutes after the u.h.f. generator was switched on there was a sharp reduction in the muscular contractions, terminating in a complete stoppage. Muscular

contractions were resumed and gradually reverted to normal when the generator was switched off. The local application of a u.h.f. field of intensity insufficient to produce muscular contraction begins to exert a stimulating action on the nerve to which it is applied if it is combined with the action of a d.c. cathode. This effect is not produced when a u.h.f. field is applied to a part of the nerve exposed to the action of an anode.

The stimulating action of a u.h.f. field may also be seen in the nerves of frogs which have been kept at low temperatures, or in a preparation in which the ionic balance has been altered by some means so that Loeb's coefficient is increased [61].

It is now apparent that a u.h.f. field alters the functional state of a myoneural preparation, but special conditions are necessary to show the stimulating effect of such a field. This is apparently the reason why Petrov [62], one of the first workers to attempt to produce muscular contraction in a myoneural preparation by exposing it to a u.h.f. field, obtained a negative result.

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A u.h.f. field alters the functional state of the myoneural apparatus. Thus, the action of curare on a myoneural preparation is weakened and sometimes even negatived when the u.h.f. field is switched on. The conditions of exposure were: MM-4 generator, power in the secondary circuit 12–17 W, wavelength 6 m, distance from electrode to muscle 3–5 mm, duration of exposure several seconds. If the generator is switched on frequently or left on for a considerable period the effect is reversed, i.e. curarization is intensified [63].

It is apparent that in any assessment of the effect of a u.h.f. field on a myoneural preparation it is essential to remember that the field influences the nerve, the myoneural endings, and the muscle itself. Attempts have been made to find the relationship between u.h.f. and the action of known parabiotic agents and to produce parabiosis by exposure to a u.h.f. field, in order to determine the nature of the action of a u.h.f. field on a myoneural preparation. Vasil'ev and Lapitskii [64] discovered that a u.h.f. field eliminated the pessimal state of a myoneural ending when used in doses which have no stimulating effect on a myoneural preparation; in this case its action resembles that of heat. The conditions of exposure are set out above.

Bilokon' and Khripko obtained similar results [65]. In their experiments exposure to a u.h.f. field eliminated a parabiotic state produced in a nerve by a low temperature (3–4°). The u.h.f. field itself acted as a parabiotic agent, and the parabiosis produced by the action of the u.h.f. was eliminated by the application of a low temperature.

In contrast to the above-named workers, Motsnyi and Reizin [66] observed no elimination of the pessimal state as a result of exposure to u.h.f. Under the conditions obtaining in their experiment muscular contractions in the u.h.f. field increased only when liminal, sub-optimal and optimal currents were used. The u.h.f. dosages used were not indicated.

Motsnyi and Reizin [66–68] concluded that a u.h.f. field may act as a parabiotic agent antagonistic to the d.c. anode and synergic with the d.c. cathode. Cathodic depression was increased by a u.h.f. field, whereas the anode's parabiotic action and the u.h.f. fields decreased reciprocally. As has already been stated, Mogendovich [61] also observed synergism in the action of a u.h.f. field and a d.c. cathode on a nerve.

The effect of a u.h.f. field on the muscular rheobase and chronaxy drew workers' attention to possible therapeutic application of u.h.f. in diseases and traumas of the peripheral nerves [69–71, etc.]. Both increases and decreases in the muscular rheobase

and chronaxy due to exposure to ultra-high frequency have been observed in clinical studies [69–75, etc.] and in experiments on animals [76–79, etc.].

Changes in the muscular rheobase and chronaxy occur when the exposure is localized in various regions—in the region of a given muscle [69, 71, 72, 76], the head [76], the head and anterior thoracic region [79], the thorax [77], etc. These phenomena are not solely connected with local functional changes, as is confirmed by changes in the rheobase and chronaxy of muscles remote from the region exposed to the u.h.f. [72, 73, 76].

THE ACTION OF A U.H.F. FIELD ON THE SYMPATHETIC INNERVATION OF THE SKELETAL MUSCLES

Motsnyi [80] studied the effect of a u.h.f. field on contractions of the calf muscle, using spinal preparations of frogs in which the sympathetic nerves on one side had been cut. When muscular contractions in response to the stimulating effect of an intermittent electric current applied to the sciatic nerve began to diminish due to fatigue, exposure to a u.h.f. field increased the extent of the contractions. This effect was apparent only on the side retaining sympathetic innervation; the nature of the muscular contractions did not alter in a u.h.f. field applied to the side on which the sympathetic nerves had been severed. Nalivaiko [81] also found that the action of a u.h.f. field on the contractions of muscles retaining sympathetic innervation differed from its action on muscles in which the sympathetic innervation was damaged.

THE ACTION OF A U.H.F. FIELD ON THE FUNCTIONS OF THE SENSORY NERVES AND RECEPTORS

It was discovered in the earliest researches into the biological effect of a u.h.f. field that such a field acts on the sensory nerves. Saidman [82] and many others studied the analgesic effect of u.h.f.; this effect is now widely used in treatment. There are also isolated references to the anaesthetizing effect of weak u.h.f. fields [17].

The marked sensitivity of the afferent nerves to u.h.f. was shown in Bekauri's experiments [22]; she discovered that exposure of a nerve to u.h.f. blocks first the afferent impulses then the efferent impulses.

Halphen *et al.* [83] explained the analgesic effect of a u.h.f. field as a reduction in the conductivity of a sensory nerve. Piontkovskii [84, 85] demonstrated that this view was incorrect; he exposed healthy persons and patients with various inflammatory processes to u.h.f. and studied the changes in tactile sensitivity and sensitivity to pain produced in them by such exposure. His results showed that local application of u.h.f. reduces tactile sensitivity. However, sensitivity to pain was not reduced; in fact it showed a certain tendency to increase. It is worth noting that even patients with inflammatory processes whose pain was reduced after u.h.f. treatment did not lose sensitivity to pain in areas close to the foci of inflammation; in fact sensitivity in these areas increased somewhat.

Exposure to a u.h.f. field of high intensity produces painful sensations. Lebedinskii [22] remarks that the nature of these pains was the same in localized exposure as in overall exposure to the u.h.f. field, basing his opinion on experiments carried out in collaboration with Glezer; the subjects of the experiments were the experimenters

themselves. For this reason the author supposed that the pains were due to action on the peripheral nerves.

Demirchoglian [86] studied the effect of u.h.f. fields on the electrical activity of a frog's retina. He discovered that a low intensity u.h.f. field, exposure to which produced no perceptible rise in temperature, caused a drop in an electro-retinograph *b* wave. The depression was reversible in the majority of cases.

The literature contains references to changes in the thresholds of vision when remote parts of the body are exposed to a u.h.f. field. Kekcheev *et al.* [87] observed an increase and a subsequent reduction in the thresholds of vision as measured from the edge of the retina when the backs and abdomens of experimental subjects completely adapted to darkness were exposed to a u.h.f. field.

The effect of a u.h.f. field on nervous trophism and the nervous regulation of the functions of organs is discussed in the preceding paper [1].

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FACTORS AFFECTING THE REACTION OF THE NERVOUS SYSTEM TO U.H.F.

Many clinicians and experimenters have remarked considerable variations in the reaction of the nervous system to u.h.f. We will not include references to all the extensive literature on this subject, confining ourselves to the reminder that irregularities in the reaction of the nervous system to u.h.f. have been observed in the course of observing persons being exposed to u.h.f. [9], in experiments on animals where various parts of the nervous system were exposed to u.h.f. [76, 88, 89] and in myoneural preparations [54, 64].

The reactions of the nervous system and its elements to u.h.f. are greatly influenced by the initial functional state of the system. Small doses of strychnine and potassium bromide to increase excitation reduce the repressive effect of a u.h.f. field on reflexes in spinal preparations from frogs. The retarding effect of a u.h.f. is intensified by the injection of large doses of potassium bromide; alcohol also greatly intensifies the retarding effect of a u.h.f. [41, 42].

Abrikosov [79] discovered that injecting rabbits with bromide and caffeine produces considerable changes in the effect of a continuous and a pulsed u.h.f. field on nervous and muscular chronaxy. A continuous u.h.f. field applied to the head and anterior part of the thorax shortened chronaxy in the fibula nerve and the tibial muscle; it either extended chronaxy in the calf muscle or left it unchanged, and produced various changes in chronaxy in the antagonist muscles. A pulsed u.h.f. field lengthened chronaxy in the fibula nerve and the tibial muscle and balanced chronaxy in the antagonist muscle. A continuous field had no effect on rabbits which had been treated with bromide and a pulsed u.h.f. field did not affect animals which were under the influence of stimulating doses of caffeine. In both the continuous field and pulsed generators the condenser plate voltage, measured by means of a Naddenko loop, was 500 V, the basic frequency was 500 Mc/s and the duration of exposure was 15 min. It was shown in experiments on dogs, using the conditioned reflex method, that the effect of u.h.f. on the functions of the cortex depended on the animal's initial physiological background [25, 89]. There was complete parallelism in one of the dogs [89] between the reaction of the cortex to the action of u.h.f. on the cerebellum and its reaction to bromide and caffeine.

The fact that reactions to u.h.f. are dependent upon the initial functional state of

the subject is also shown by the experiments on a myoneural preparation described by Mogendovich, Vasil'ev and Lapitskii [54, 64, etc.].

When exposure to u.h.f. is repeated, the effect alters. In such circumstances both increases and decreases in the efficacy of exposure are possible. Zhukhin [90] remarks that morphological changes occurring in the central nervous system as a result of chronic u.h.f. field effects are less extensive than those arising after severe exposure.

Conditioned reflex activity in dogs is affected less by repeated applications of u.h.f. to the cerebellum and the cortex than by the first application. In this case, however, it has been observed that repeated exposure to u.h.f. intensifies the effect on the functions of the nervous system. In one series of experiments the subject was a dog with remarkable resistance to u.h.f.; a u.h.f. field of strength 45–60 W was applied to the cerebellum region daily, the duration of each exposure being 10–20 min. The first four doses produced no effect, but the conditioned reflexes were reduced after the fifth dose; on the next day, however, normal conditioned reflex activity was restored, and nine more daily applications of the same dose produced no further changes in conditioned reflex activity [89]. There are evidently two tendencies to consider when giving repeated doses: a tendency for the effects to intensify and a tendency for them to decrease. The final result depends on which of these tendencies will prevail at a given stage.

CONCLUSION

The analysis of the physiological mechanism of u.h.f. effects on the functions of various sections of the nervous system is extremely complex. In making such an analysis it is essential to allow for possible direct action of the u.h.f. on the nerve centres, for general and localized changes in the circulation of the blood produced by these factors, for changes in the spinal fluid pressure and for dermal and muscular reflexes, etc. Exposure to powerful fields also produces pain.

Glezer [91] studied conditioned reflex activity in dogs exposed to u.h.f. fields of high intensity, accompanied by a rapid rise in body temperature and marked defensive reactions (the physical characteristics of the u.h.f. field used are not given). Under such conditions the pain effect is dominant. When a dog in which conditioned feeding and defensive reflexes had been induced was exposed to such a field the former reflexes were retarded but the latter were retained. This is not a specific reaction to exposure to u.h.f.; it also occurs when other excessive stimuli are applied. However, much information about the effect of u.h.f. on the function of the cortex was obtained by using doses which produced no perceptible defensive reactions in the animals [23, 26]. Experiments in which various methods were used confirm that the effects of u.h.f. depend on the area which is exposed to it. Changes in the critical frequency of image-merging occur when it is applied directly to the eyes, but no such changes were recorded as a result of bitemporal application [33].

When the auditory zones of the cortex of a strong type dog were exposed to a u.h.f., conditioned reflex changes were confined to the sound analyser, whereas similar treatment of the frontal lobes and cerebellum causes changes in conditioned reflex activity which affect various analysers [89]. The fact that the reactions depend on the area exposed to the u.h.f. indicates that the sensations of pain, general changes in cerebral blood circulation and intracranial pressure and skin and muscular reflexes produced by the small u.h.f. doses used by the authors mentioned had no decisive

significance; the fundamental factors here were changes in the functional state of the appropriate nerve centres.

The question whether the functional changes produced by a u.h.f. field are the result of its direct action on the nerve tissue or a consequence of local vascular changes has not been studied experimentally. It is most probable that both these factors contribute to the changes in question. When any agent acts on the nervous system two types of effect may be produced; non-specific effects, which can be produced by any agent acting on a given part of the nervous system, and the specific effects of the type of influence used.

There is no doubt that u.h.f. radiations have a non-specific effect on the nerve centres. One of these non-specific effects is the phenomenon of Sechenov inhibition which can be produced by other means [38]; the similarity between some of the reactions of the nervous system to local application of a u.h.f. field and its reaction to ionizing radiation [89] also indicates that non-specific u.h.f. effects are possible. However, the specific effects of this particular physical factor must not be underestimated. Abrikosov's detailed and systematic research [79] is especially interesting in this respect; he showed that there are marked differences between the reactions of the nervous system to a continuous u.h.f. field and its reaction to an impulse field, conditions of exposure being the same in both cases.

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THE TEMPERATURE OF PLANT LEAVES ON ARTIFICAL ILLUMINATION*

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THE energy balance of a plant leaf contains terms relating to absorption of radiant energy and to energy consumption in heat loss transpiration and photosynthesis [1, 2]. This subject has so far been largely neglected for plants subject to artificial illumination [2–9]. A normal leaf on a higher plant can be considered as a plate of thickness negligible in relation to its other dimensions. Neglecting transpiration, the rise in leaf temperature $d(\Delta t)$ on illuminating for an infinitessimally short time $d\tau$ is given by

$$\frac{d(\Delta t)}{d\tau} = \frac{Q\alpha}{mc} - \frac{2k_i}{mc}\Delta t \tag{1}$$

where m is the mass per unit area (g/cm²), c is the thermal capacity, on average $3{\cdot}59 \times 10^7$ ergs/g°C, Q is the incident light intensity (ergs/cm² sec), α is the fraction of the incident energy absorbed (as a percentage), k_i is the heat transfer coefficient (ergs/cm² sec°C) and Δt is temperature difference between leaf and air (°C). The integral of (1) gives the time-variation of the temperature excess in the leaf as

$$\Delta t = \frac{Q\alpha}{2k_i}(1 - e^{-2k_i\tau/mc}). \tag{2}$$

Thus Δt is directly proportional to Q and α and inversely proportional to k_i, and equilibrium is reached more quickly the lower m and c, or the higher k_i. Equation (2) shows that Δt is in theory zero in darkness and tends as $\tau \to \infty$ to some limit Δt_m given by

$$\Delta t_m = Q\alpha/2k_i. \tag{3}$$

For practical purposes, Δt_m is reached in a time τ given by

$$\tau = 2{\cdot}3\, mc/k_i. \tag{4}$$

Calculations show that leaves of *Datura arborea*, *Ilex pyramidalis*, *Begonia rex* and *Acer platanoides*, which have average m values of 0·031 g/cm², reach equilibrium in 4 min. The error in Δt is then not more than 1 per cent. Equation (2) also implies that at equilibrium (with transpiration absent) the entire absorbed energy $Q\alpha$ is lost by heat transfer (i.e. $Q\alpha = 2k_i\Delta t$).

Fig. 1 shows the theoretical Δt curves calculated from (2) for *Acer platanoides* (m=0·023 g/cm²) and *Gedum sp.* (m=0·085 g/cm²), and the experimental curves. The experimental curves always lie below the theoretical, and the Δt_m is reached the sooner the thinner the leaf. With low Q (or at any intensity with succulents) there is

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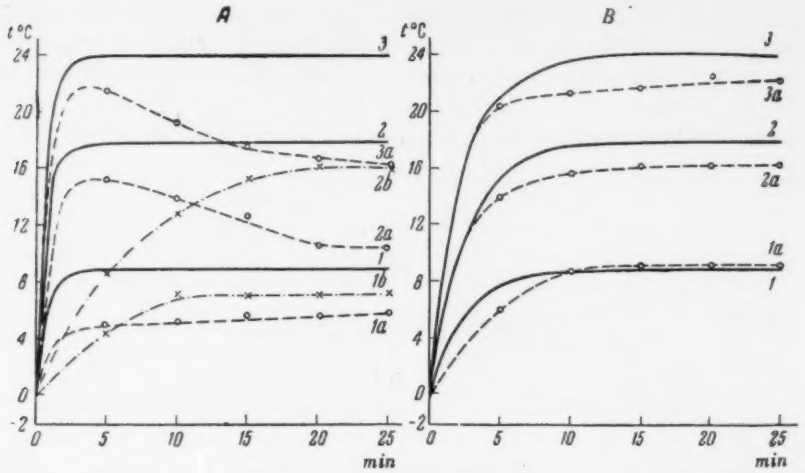

FIG. 1. Variation of Δt with time for plants illuminated by incandescent lamps with reflectors. (*A*) *Acer platanoides;* (*B*) *Sedum sp.* (1) $3{\cdot}5\times10^5$, (2) $7{\cdot}6\times10^5$, (3) 10^6 (ergs/cm² sec). Changes found by experiment: (1*a*) $3{\cdot}5\times10^5$, (2*a*) $7{\cdot}0\times10^5$, (3*a*) 10^6 (ergs/cm² sec). Changes found with yellow autumn leaves: (1*b*) $3{\cdot}5\times10^5$, (2*b*) $7{\cdot}0\times10^5$ (ergs/cm² sec).

no major difference between the curves. With high Q the experimental curves for normal thin leaves are decidedly different from the theoretical ones. The theoretical one reaches a steady level, according to (3), while the experimental runs parallel to the theoretical for 3–4 min, and then falls to a lower level, Δt_s, which becomes steady after 20–25 min [7–9]. The differences are particularly marked with tin-leaved mesophytes and high transpiration rates.

These effects only occur with living leaves; Table 1 gives the absolute values of Δt, while Fig. 2 shows the time variations for live leaves of *Ilex pyramidalis* and *Malus communis*, and for leaves killed in various ways. For dead leaves Δt is clearly 6–11°C above that for live ones. The dead leaves follow (2); the only difference is that dead leaves containing water (*Malus communis*) vary much more slowly than do dry ones (*Ilex pyramidalis*). This occurs because the water evaporates; the effect is seen particularly clearly with the yellow autumn leaves of *Acer platanoides* (Fig. 1*A*).

TABLE 1. Δt VALUES FOR LIVE AND DEAD LEAVES ILLUMINATED BY INCANDESCENT LAMPS WITH REFLECTORS AT AN INTEGRAL RADIATION INTENSITY OF 10^5 ERGS/CM² SEC

Type	Steady-state Δt (°C)
Ilex pyramidalis	
Normal green leaf	16·7
Dry leaf	22·5
Acer platanoides	
Normal green leaf	15·8
Yellow autumn leaf	22·0
Malus communis	
Normal green leaf	17·8
Leaf killed with ether	28·4
Autumn leaf after frost (November)	29·7

It was found that Δt for dry and wet cambric followed (2) strictly, so simple evaporation does not alter the shape of the curve (Fig. 3). When dry cambric with its ends inserted in water was used, however, the curve for a live leaf was reproduced (Figs. 3, 2a). As the cambric takes up water, which evaporates, Δt falls. The shape of the curve for live leaves is thus caused by transpiration; the Δt curves for leaves

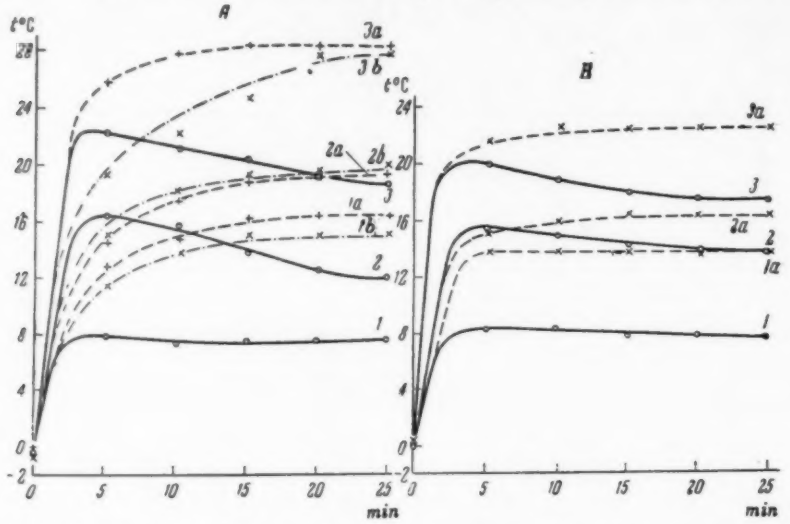

FIG. 2. Variation of Δt with time for plants illuminated by incandescent lamps with reflectors. (*A*) *Malus communis*. Normal green leaves: (1) $3{\cdot}5\times10^5$, (2) $7{\cdot}0\times10^5$, (3) 10^6 (ergs/cm² sec). Leaves killed with ether: (1*a*) $3{\cdot}5\times10$, (2*a*) $7{\cdot}0\times10^5$, (3*a*) 10^6 (ergs/cm² sec). Autumn leaves killed with ether: (1*b*) $3{\cdot}5\times10^5$,(2*b*) $7{\cdot}0\times10^5$, (3*b*) 10^6 (ergs/cm² sec). (*B*) *Ilex pyramidalis*. Normal green leaves: (1) $3{\cdot}5\times10^5$, (2) $7{\cdot}0\times10^5$, (3) 10^6 (ergs/cm² sec). Dry leaves: (1*a*) $3{\cdot}5\times10^5$, (2*a*) $7{\cdot}0\times10^5$, (3*a*) 10^6 (ergs/cm² sec).

of different ecological types, anatomical structures, colours, transpiration rates, etc., are of some interest in this connexion. The plants listed in Table 2 were used with filament lamps fitted with reflectors at various integral radiation intensities and air temperatures varying from 19–22° to 22–26°C during the experiment (lasting 25 min). The temperature was determined by the method previously described [7, 9]. Cut twigs or leaves with their lower ends or stems plunged in water were used. This technique [2] eliminates root effects, though the only difference is that leaves remaining on the plant show Δt values some 10–15 per cent lower.

Table 2 and Figs. 4–6 show the results. In most cases Δt rises rapidly, and becomes constant after 3–4 min at low light intensities (Fig. 4*A*), while at high intensities a fall begins after the maximum, a new low level being reached after 25–30 min (Fig. 4*B*). The fall depends on the thickness of the leaf and on the type of plant. The thicker and more mesomorphous the leaf the larger and more rapid the fall (*Datura arborea*, Fig. 4*B*, 1) and conversely (*Ficus parcelli*, Fig. 4*B*, 3). No fall is found at all with succulents (*Gedum sp.*, Fig. 4*B*, 4). Withering usually leads to a late rise in Δt (*Begonia rex*, Fig. 4*B*, 2). The fall is thus clearly caused by transpiration [2, 10] and depends on the type of plant. Fig. 5 shows the differences

between the maximum and steady Δt values vs. Q; in most cases a difference is seen at 350,000 ergs/cm² sec which then increases with Q, becoming maximal at 700,000 ergs/cm² sec. Further increase in Q has no effect. No difference is found in the

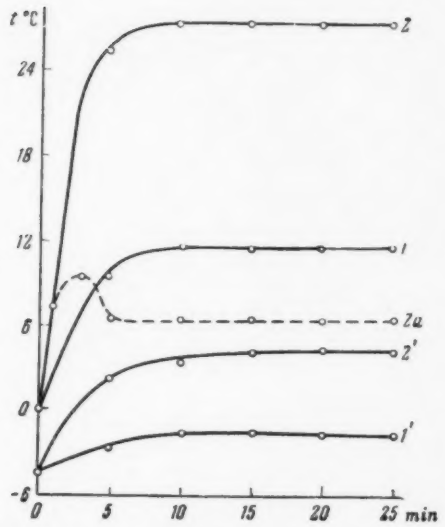

FIG. 3. Variation of Δt with time for dry leaves (1, 2) and wet cambric (1′, 2′) illuminated with incandescent lamps with reflectors. (1) $3{\cdot}5 \times 10^5$, (2) 10^6 (ergs/cm² sec). (2*a*) dry cambric gradually moistened (at 10^6 ergs/cm² sec).

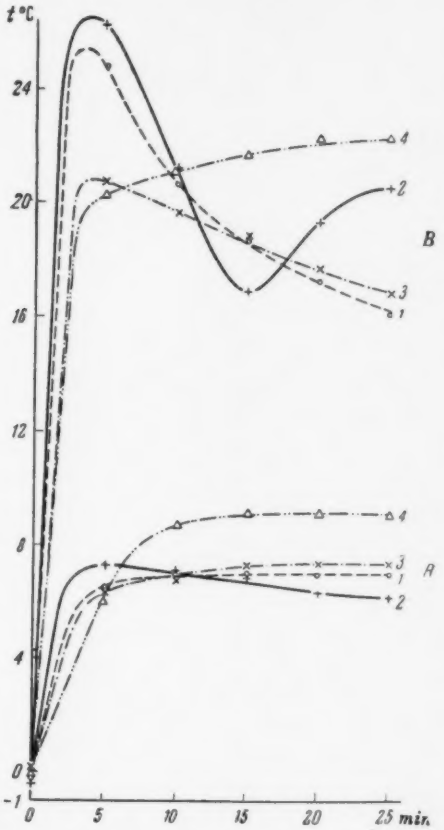

FIG. 4. Variation of Δt with time for plants illuminated by incandescent lamps with reflectors. (*A*) at $3{\cdot}5 \times 10^5$ ergs/cm² sec, (*B*) at 10^6 ergs/cm² sec. (1) *Datura arborea*; (2) *Begonia rex* (red); (3) *Ficus parcelli* (green leaf); (4) *Sedum sp.*

succulents (*Gedum sp.*, *Euphorbia grandii*); the value rises from 2 to 4°C in the xerophytes (*Ficus parcelli*, *Ilex pyramidalis*, etc.) to 8–10°C in meosphytes (*Datura arborea*, *Solanum lycopersicum*, etc.).

Table 2 gives the Δt values, and Fig. 6 how they vary with Q. It is clear that Δt increases in going from mesophytes to xerophytes, and is maximal in the succulents. Chlorophyll, anthocyanins and flavones also increase Δt, if present, as the examples of *Ficus parcelli*, *Begonia rex*, *Acer platanoides*, *Acer rubrum* and *Populus tremula* show. For light leaves Δt is higher than for dark (*Malus communis*, *Salix sp.*, *Convallaria majalis*), and it is also higher for old leaves (*Ilex pyramidalis*). If the water

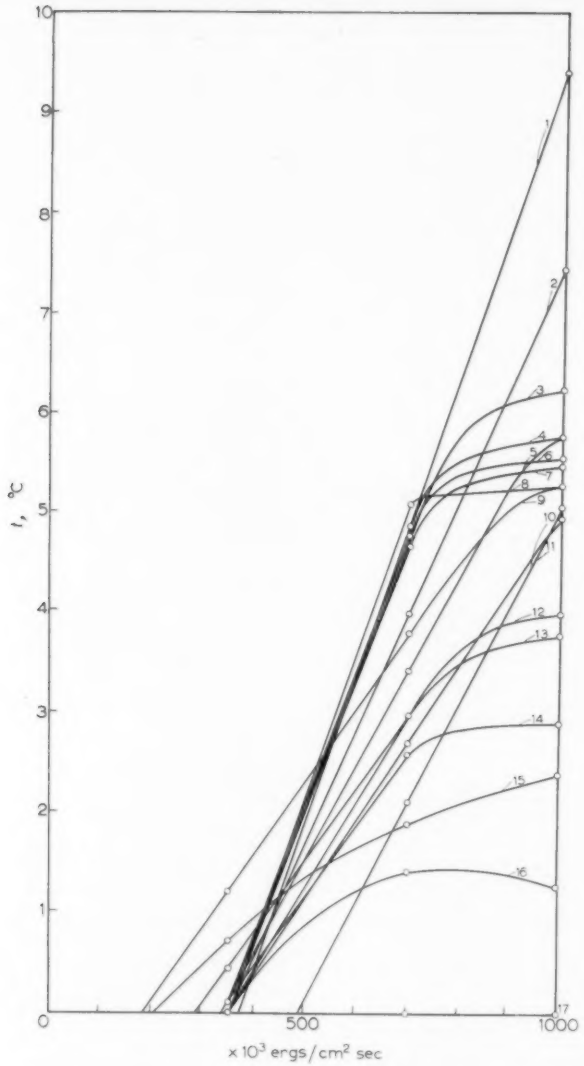

FIG. 5. Variation of the difference between the maximal and steady-state temperature rises ($\Delta t_m - \Delta t_s$) with the light intensity. (1) *Datura arborea*; (2) *Solanum lycopersicum*; (3) *Hydrangea bortensis*; (4) *Malus communis*; (5) *pulmonaria officinalis*; (6) *Cucumis sativus*; (7) *Acer platanoides*; (8) *Acer rubrum*; (9) *Salix sp.*; (10) *Pirus communis*; (11) *Asarum europaeum*; (12) *Fiscus parcelli* (green leaf); (13) *Calla ethiopica*; (14) *Begonia rex* (green leaf); (15) *Ilex pyramidalis*; (16) *Ficus parcelli* (white leaf); (17) *Euphorbia grandii*, *Gedum sp.*

supply is normal and withering is absent, Δt_s is directly proportional to Q (Fig. 6). Some 45,000–68,000 egrs/cm² sec are required to raise the temperature of normal green leaves, or ones containing anthocyanins by 1°C (Table 2). The value is maximal in mesophytes, lower in xerophytes and least in succulents, and is on average 58,500 ergs/cm² sec.

TABLE 2. Δt VALUES FOR LEAVES ILLUMINATED AT AN INTEGRAL RADIATION INTENSITY OF ERGS/CM² SEC

Plant	Δt_m (°C)	Δt_s (°C)	Integral intensity which increases Δt by 1°C × 10⁻³ (ergs/cm² sec)	Illumination which increases Δt by 1°C (lux)
Sedum sp.	22·2	22·2	45·0	650
Euphorbia grandii	21·5	21·5	46·6	675
Calla ethiopica	24·0	20·2	49·5	715
Asarum europaeum	22·7	17·7	55·4	800
Datura arborea	25·5	16·0	57·5	825
Cucumis sativis	21·4	15·8	58·0	835
Pulmonaria officinalis	21·5	15·7	62·8	905
Pirus communis	20·5	15·4	65·0	935
Solanum lycopersicum	22·7	15·2	65·8	945
Hydrangea hortensis	21·3	15·0	66·8	960
Ficus parcelli				
Green leaf	20·7	16·7	60·0	870
White leaf	13·3	12·0	83·5	1200
Begonia rex				
Green	18·6	15·7	63·8	920
Red	26·3	20·4	49·0	710
Acer platanoides	21·3	15·8	63·4	920
Acer rubrum	21·7	17·4	57·5	830
Populus tremula				
Green leaf	19·5	15·9	63·0	910
Brown leaf	20·7	16·6	60·3	870
Malus communis				
Light	23·4	17·6	56·8	820
Dark	22·3	18·7	53·5	775
Salix sp.				
Light	20·3	15·0	66·8	965
Dark	22·3	17·7	56·5	815
Convallaria majalis				
Light	20·3	16·3	61·5	890
Dark	23·3	18·3	54·6	790
Ilex pyramidalis				
Young leaf	17·8	14·7	68·0	980
Old leaf	20·0	17·6	57·0	825

The heat transfer coefficient was determined by the method of Brown and Wilson [2, 11] for 15 types of plants with very different types of leaves. These results, together with Δt and transpiration rate in darkness at an air temperature of 19–22°C, are given in Table 3. The k_i vary from $8{\cdot}9 \times 10^3$ to $13{\cdot}2 \times 10^3$ ergs/cm² sec °C, with a mean of $10{\cdot}5 \times 10^3$, which agrees with the data of Brown and Wilson [10]. The differences between species are due solely to technical difficulties in the determination of quantities such as the temperature difference and the true transpiration rate in darkness. One

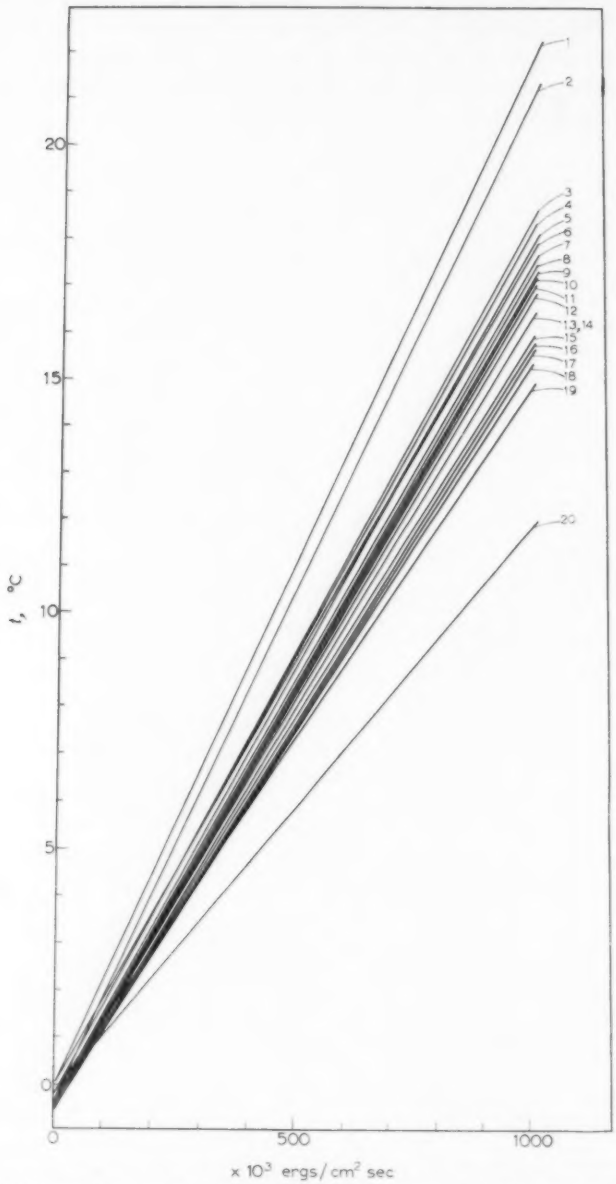

FIG. 6. Variation of Δt with the integral radiation intensity. (1) *Sedum sp.*; (2) *Euphorbia grandii*; (3) *Malus communis* (dark plant); (4) *Convallaria majalis* (dark plant); (5) *Asarum europaeum*; (6) *Ilex pyramidalis* (old leaf); (7) *Malus communis* (light plant); *Salix sp.* (dark plant); (8) *Datura arborea, Cucumis sativus, Acer rubrum*; (9) *Solanum lycopersicum*; (10) *Ficus parcelli* (green leaf); (11) *Populus tremula* (brown leaf); (12) *Calla ethiopica*; (13) *Pirus communis*; (14) *Convallaria majalis* (light leaf); (15) *Ilex pyramidalis* (young leaf); (16) *Populus tremula* (green leaf); (17) *Acer platanoides*; (18) *Pulmonaria officinalis*; (19) *Salix sp.* (light leaf); (20) *Ficus parcelli* (white leaf).

TABLE 3. k_i FOR LEAVES OF VARIOUS PLANTS

Plant	Leaf area (cm^2)	Transpiration rate $\times 10^6$ (g/cm^2 sec)	Δt (°C)	k_i (ergs/cm^2 sec °C)
Sedum sp.	50·0	0·09	−0·12	8900
Euphorbia grandii	48·1	0·14	−0·18	9700
Calla ethiopica	50·0	0·40	−0·50	9700
Asarum europaeum	52·3	0·27	−0·36	9100
Datura arborea	251·3	0·22	−0·24	11400
Pulmonaria officinalis	46·5	0·45	−0·50	10900
Hydrangea hortensis	42·3	0·33	−0·36	11000
Gossipium hirsutum	—	0·63	−0·73	10400
Ficus parcelli				
Green leaf	41·6	0·23	−0·24	10800
White leaf	36·4	0·19	−0·24	9600
Begonia rex				
Green	100·0	0·42	−0·40	12700
Red	51·0	0·36	−0·48	9100
Greeny-red	95·0	0·39	−0·36	13200
Acer platanoides	75·9	0·37	−0·36	12400
Acer ruburm	57·4	0·28	−0·36	9400
Malus communis				
Light	55·0	0·50	−0·48	12600
Dark	61·0	0·40	−0·48	10000
Convallaria majalis				
Light	112·6	0·48	−0·60	9700
Dark	104·7	0·40	−0·48	10100
Ilex pyramidalis				
Young leaf	45·0	0·30	−0·36	10300
Old leaf	50·1	0·14	−0·18	9400
Mean	—	0·33	−0·38	10500

important source of error is that the temperature of a leaf is not everywhere the same [12].

SUMMARY

(1) The temperature excess of plant leaves artificially illuminated in still air rises rapidly at the start of illumination, is maximal after 4 min, and then falls somewhat to a constant level in 25–30 min.

(2) This applies only to live leaves. Dead, dry, or yellowed leaves behave like dry or wet cambric, and give an exponential curve.

(3) The difference between the maximum and steady temperature excess values depends on the type of plant, being zero for succulents, quite small for xerophytes and maximal for mesophytes.

(4) The temperature excess is least for mesophytes, greater for xerophytes and greatest for succulents.

(5) The excess is higher in dark and shade-loving plants. Leaves containing flavones and anthocyanins also show higher values than normal green leaves.

(6) The excess is directly proportional to the integral radiation intensity. A radiation intensity of about 60,000 ergs/cm^2 sec is required to raise the temperature

of a leaf adequately supplied with water by 1°C. This corresponds to 845 lux for filament lamps.

(7) The heat transfer coefficient is independent of leaf anatomy and colour and is 10,500 ergs/cm² sec °C.

Translated by J. E. S. BRADLEY

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DIFFUSIBLE TOXIC COMPOUNDS IN RADIATION DAMAGE IN PLANTS*

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AN important feature of radiation damage mechanisms which requires elucidation is the power of biologically active materials to diffuse freely from damaged areas. It has been demonstrated that some such materials with toxic properties can be transferred humorally in animals [1–3]. For plants it has not yet been shown whether the damage remains localized in the cells, or whether some readily diffusible toxin of low molecular weight (which may stimulate at low concentrations) causes the damage to spread. The second idea is supported by King's [4] *in vitro* work on tobacco calluses, which showed that some growth inhibitor could diffuse through the solution from an irradiated part to an adjacent normal one. The same conclusion is indicated by the work of Skorodinskaia and Dubovaia [5], which showed that seeds of the Australian pea germinated more rapidly when placed in very dilute water extracts of X-irradiated aloe leaves. Heilbrunn and Daugherty's work [6] on the effects of ultraviolet light on *Elodea* leaves and Kaplan's [7] on irradiated soya bean seeds point to the same conclusion. On the other hand, Smith and Kersten [8] found no toxic materials in water extracts from irradiated pea seeds. Murray's [9] and Christensen's [10] studies of irradiated sections of plants also indicate that no diffusible materials are produced.

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The problem is therefore at present unresolved. Our work was directed to determining whether these apparent contradictions are accidental or not, and if the latter, whether the effects are necessarily due to "radiation toxins".

METHODS

The detector seedlings were placed in media in which the active materials produced by irradiation were supposed to exist. The growth rate and overall length of the roots were used as indices. This test was used because cell division (and hence growth also) is very sensitive to irradiation. Seedlings of *Vicia faba* were mainly used; in some cases cucumber, sunflower, and hemp seedlings were used. The seedlings were either grown from the dry seeds, or else were ones which had previously stood for a certain time in the solution. Their ages at the ends of the tests varied from 4 to 9 days. The bean seedlings were grown in moist sawdust prior to use, and afterwards in test-tubes, the cucumber ones on moist filter paper in petri dishes, and the others on moist paper pads. The temperature was at all times kept constant at 24–25°C. The water extracts were prepared by mashing weighed amounts of the seedlings with water in a mortar followed by filtration through two layers of muslin. Tap water was usually used, though on occasion distilled water was substituted.

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The bean seedlings were irradiated on a URS-70 X-ray unit run at 14–15 mA, 100 kV, with filters of cardboard or 0·6 mm Al, and at a focal distance of 1 cm. The dry seeds, and seedlings of other plants, were irradiated with ^{60}Co γ-rays in a GUT-400 unit at dose rates from 100 to 500 r/min.

RESULTS AND DISCUSSION

The effects of direct irradiation and of any substances produced were first studied in terms of the effects on the growth of irradiating dry seeds and seedlings. With the bean seedlings it was found that macroscopically observable growth rate changes occurred not at once, but after 2 days. Irradiations lasting 15 and 45 sec only halted rootlet growth temporarily, while ones lasting 1 and 3 min disturbed the growth permanently. It was also found that the types of growth inhibition produced with dry seeds and day-old seedlings were identical. Small doses produced a sharp drop in rootlet length, while at higher doses the smooth dose curve usually found was obtained. It was found that the region of greatest slope for γ-rays acting on seedlings fell between 500 r and 2000–5000 r, and between 1000 and 10,000 r for dry seeds. In the next series standard γ-ray doses of 1500 r were used with dry seeds, and ones which had been in contact with water for 1, 6, 12, 24, 48 and 72 hr. The beans and hemp were found most sensitive at 48 and 72 hr, the cucumbers at 24 hr. The subsequent rises in sensitivity were slight. We therefore used bean shoots 48 and 72 hr old and cucumber ones 24 hr old in our tests.

The biological activities of root extracts from irradiated seedlings were studied in the first set of experiments. Normal bean seedlings 2 days old were placed in a medium in which seedlings irradiated for 5, 10 and 15 min, or not irradiated (control) had previously been grown for 6–8 days. A "complete control", using pure water only, was also run. Fig. 1 shows the rootlet growth curves; all are of the same shape. Some difference between the final lengths is seen, however, particularly with the

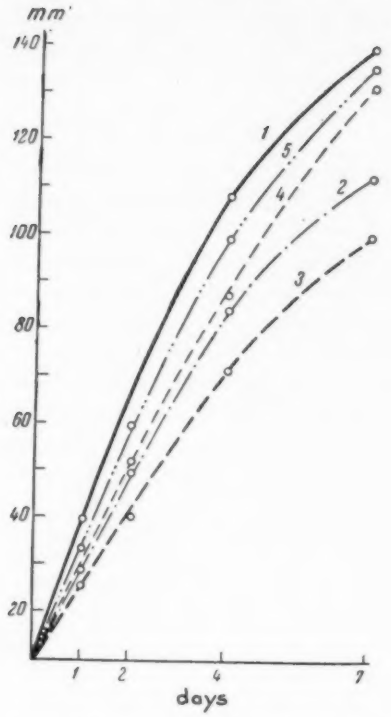

FIG. 1. Abscissa: time grown on medium (days); ordinate: root length (mm). (1) Full control (water), (2) control (water from normal seedlings), (3) 5 min irradiation, (4) 10 min, (5) 15 min. There were 6–7 plants in each group. One of 5 similar runs is shown.

5 and 15 min irradiations. This result was checked by using a large batch of plants. Table 1 gives the results; the divergence between the experimental and control plants only slightly exceeded the experimental error.

The cause of the slight effect may have been that the concentrations were too low. We therefore repeated the tests using five sets of forerunners for 5–6 days each, instead of one only. In one series the detectors were also given small X-ray doses prior to use. However, the effects were in all cases very slight. The slight stimulation effect found in the first group (Table 1) is therefore to be explained as due to slight differences

TABLE 1. ROOTLET LENGTHS IN IRRADIATED PLANTS AND DETECTOR PLANTS

Irradiation time of forerunners	Rootlet lengths after 7 days (irradiated plants)		Rootlet lengths of detectors (m + mm)	
	mm	%	Initial	After 6 days
0 (full control)	—	—	27·1	158 ± 5·7
0 (control)	163	100	28·8	135 ± 4·95
5 min	49	30	29·9	153 ± 3·3
15 min	39	24	28·6	152 ± 5·4

in oxygen concentration in the tubes, and not to diffusible stimulants. This could have occurred because the irradiated roots were some 70 per cent shorter than the normals, and so occupied less of the test-tubes.

The next series dealt with the biological activity of aqueous extracts of irradiated seedlings. If it is difficult for the active materials to escape through the cell walls, as has been demonstrated for animals [11], the intracellular concentrations ought to be higher. In these tests various values were taken for the γ-ray dose (50–5000 r), the time from irradiation to extraction (1 hr to 5 days) and the dilution of the extract (1: 5 to 1: 100). Bean, sunflower and cucumber seedlings were used, in a total of 26 series. The results were similar to those given in Tables 2 and 3 (^{60}Co γ-rays at 500 r/min were used); the detectors were cucumber seedlings. The "experimental" plants were grown in extracts from irradiated seedlings, the "controls" in ones from normal seedlings. As before, the differences between the two sets were slight and fell within the limits of experimental error.

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TABLE 2.* SEEDLING LENGTH IN IRRADIATED PLANTS AND DETECTOR PLANTS

Dose (kr)	Lengths of irradiated seedlings after 6 days		Lengths of 5 day detectors	
			Normal	Given doses of 800 r before placing in extract
	mm	%	mm ± m	
0 (full control)	110·1	100	92·0 ± 2·16	71·0 ± 3·72
0 (control)	—	—	90·6 ± 2·49	71·0 ± 2·61
1	93·9	85	85·0 ± 2·48	81·0 ± 2·46
5	41·5	37·7	88·5 ± 2·78	80·0 ± 2·66
10	29·7	27	84·5 ± 2·99	81·0 ± 2·50
20	24·8	22·6	78·5 ± 2·45	71·0 ± 2·40
50	22·7	20·6	84·3 ± 2·60	75·9 ± 2·94

* The values are the means from two series of 20 plants each. The 1: 10 extracts were made from cucumber seedlings 1 day after irradiation. The 1 day old detectors were grown on the extracts for 2 days.

The results given in Table 3 show that the lengths were practically unaffected by the time the seedlings were left in the extract, which shows that no biologically active material occurred. The lengths of the controls on extract were greater than for those on pure water at 18 hr, but appreciably less at 72 hr. This is probably because growth stimulants are destroyed by irradiation [8, 12–14].

This lack of effect indicates that the biologically active materials (if produced) are very labile, and very rapidly break up on leaving the cells. We therefore set up a series of experiments in which normal cucumber seedlings were alternated on petri dishes with ones irradiated with ^{60}Co γ-rays in such a way that the roots were in close contact. Doses of 0 (control) and 1, 5, 10, 20 and 50 kr were used; the lengths of the roots did not differ by more than the experimental error. It merely remained to see whether the "radiation toxins" were capable of getting through cell walls at

TABLE 3.* LENGTHS OF 4 DAY SEEDLINGS VS. TIME GROWN ON EXTRACT

In	Time on extract (hr)						
	0	18		28		72	
	Full control	Control	Experiment	Control	Experiment	Control	Experiment
mm	107·0±1·6	116·0±1·85	96·8±2·1	98·7±2·3	94·5±2·7	91·0±4·0	93·0±1·6
%	100	108·5	90·5	92·1	88·3	85·0	87·0

* The values are the means for 20 seedlings. The 1: 5 extracts were prepared from sunflower seedlings 1 hr after irradiation at 50,000 r.

all, and to do this we irradiated small areas of the roots of 3 day bean seedlings some 5–7 mm from the base. The upper and lower areas of the roots were screened from the X-rays with lead tubes. The mean lengths 6 days later were 92±6·7, 93±6·8 and 97·5±4·3 for the controls, 10 min irradiated and 60 min irradiated plants respectively, i.e. there was no effort. Fig. 2 shows that the experimental and control plants showed marked differences. No rootlets appeared on the irradiated areas, which were also somewhat thinner than in the controls. The stem lengths were 100±2·7 per cent (control) and 85·3±4·5 per cent (irradiation at the middle of the root; if the tips were irradiated the value was 81±4·9 per cent). This effect did not prove that the growing point was affected by toxins, however, since a special test (Table 4) showed that the stem length was independent of the dose. If toxic

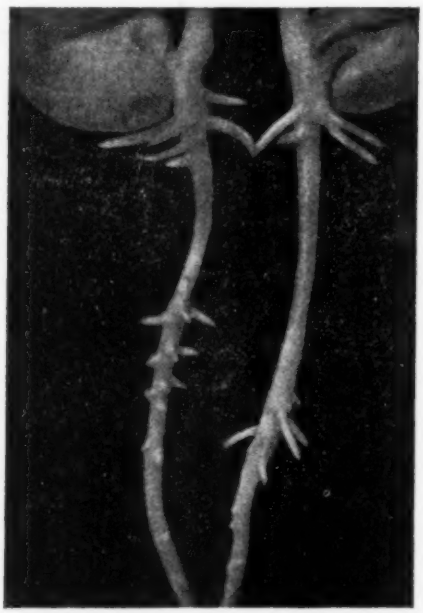

FIG. 2. The main roots of 5 day old bean seedlings 3 days after irradiation. The middle of the root on the right has been irradiated.

TABLE 4.* DEPENDENCE OF STEM LENGTH ON DOSE OF RADIATION

Irradiation time (min)	Stem length (mm)
0	160·0
5	135·2
30	141·0
60	134·5

* The results from one of five sets of similar experiments are given.

substances were responsible the effect should be dose-dependent. The cause is probably reduced water supply from the irradiated roots (the rootless were fewer and the root diameter somewhat smaller).

Fig. 3 shows that the area in the root free of rootlets increases with the irradiation time; this is caused by the scattering of the X-rays at the edge of the screening tubes, and need not be diffusible materials. This was checked using bean seedlings with the root tips placed 3 mm from the edge of the screen, using a 60 min irradiation; the roots were only 64·5 per cent of the length of the controls after 6 days. All our results thus indicate that toxic materials are either not formed, or if formed are not soluble in water, with bean, cucumber and sunflower seedlings. This agrees with the results of others. Murray [9] found that lucerne scions X-irradiated at up to

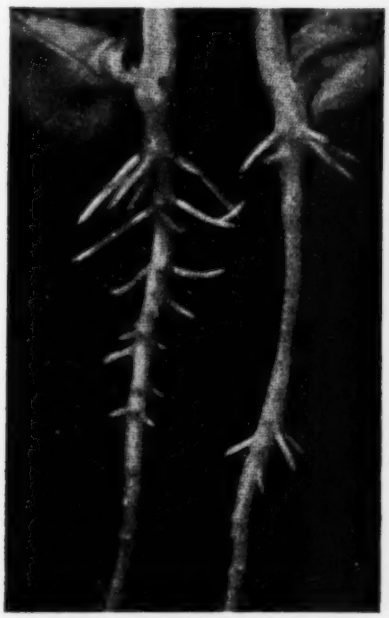

FIG. 3. The main roots of bean seedlings 4 days after irradiation. The middle of the root on the left was irradiated for 5 min, the middle of that on the right for 60 min.

18,000 r (with the lower part screened) formed roots in just the same way and of just the same lengths as control scions. Christensen [10] found just the same with *Xanthium*, tobacco and tomato plants on irradiating small areas of the stems at doses up to 24,000 r. Some authors have also drawn contradictory conclusions from their observations [4–7], although the experiments were undoubtedly done carefully. We therefore conclude that substances of toxic properties and low molecular weight which are stable and mobile by no means always form in irradiated plants of all types. The common damage process may occur in the cellular proteins; the high radiosensitivity of proteins is well known. The effect may be a disturbance to normal metabolism, and some of the abnormal products may well be toxic. This is probably the explanation of the result of [4–7].

SUMMARY

The preliminary experiments showed that X-irradiation of bean root tips took 2–3 days to produce a macroscopically observable decrease in growth rate. Whether the effect was reversible or not depended on the dose. ^{60}Co γ-irradiation of dry seeds and seedlings of cucumbers, beans, hemp and sunflower produced a reduction in root-hair and stem length varying exponentially with dose; the slope of the dose curve was greatest at 500–5000 r for 1 day old seedlings, and 1000–10,000 r for dry seeds. The sensitivity to ^{60}Co γ-rays also varied exponentially as a function of soaking time. The conclusions from the experiments on the indirect effects of radiation (i.e. on substances produced by irradiation) are as follows.

(1) No biologically active substances were observed to be transferred between irradiated and normal bean seedlings grown in succession in the same water. No such substances were found in water extracts of irradiated seedlings either.

(2) Normal seedlings were not affected by being grown side by side with irradiated ones.

(3) Narrow-beam X-irradiation of the central root areas in bean seedlings did not affect the overall lengths of the roots 6–7 days later, but the stems were somewhat shortened.

(4) The destruction of growth stimulants by irradiation was confirmed.

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INCREASES OF DRY WEIGHT IN WHEAT LEAVES AFTER X-IRRADIATION*

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It has been shown [1] that X-ray doses of 10^4 r to wheat seedlings eliminate the meristem tissue in the root-tips, but that the cells remain viable, and only develop large vacuoles. The same has more recently been demonstrated for leaf meristem tissue. The numbers of vacuolized cells increase with the dose (in the 500–1000 r range); all are vacuolized at 3000 r. This is well seen from the root hairs formed in plants grown in water culture; after 3000 r doses the entire roots are thickly covered with hairs, whereas this is never found in the meristem zone with normal plants. In leaves the vacuolization is indicated by the "sprouting", which usually lasts a week (Fig. 1). This vacuolization is supposed to be caused by the growth being depressed, so that substances which should be used up instead accumulate. The fact that the meristem cells vanish is itself proof that growth is depressed. The present paper gives the evidence for unused material accumulating.

METHODS

We used type 599 winter wheat, obtained from the Institute of Wheat Farming for the Non-Black-Earth Region. Selected seeds of uniform size were soaked in tap water, and after incubation for 24 hr in a thermostatic oven were spread out on Perspex grids in Koch dishes supplied with Mayer's solution. The seedlings were kept in an artificially lit box at constant light intensity at $23 \pm 2°C$. After 4 days the seedlings were irradiated with a RUM-3 unit, at 15 mA, 180 kV, focal distance 17 cm, dose-rate 530 r/min. The dry weight was determined by drying the leaves to constant weight at 105°C.

RESULTS

The percentage content of dry matter in the leaves was determined as follows.

(1) The plants were cut on the day of irradiation. The new growth formed in the next 7 days was used; by this time the vacuolization was complete in all plants given doses of 0·3, 0·5 and $1{\cdot}0 \times 10^4$ r (Fig. 1).

Control	1000 r	3000 r	5000 r	10,000 r
$9{\cdot}0 \pm 0{\cdot}50$	$10{\cdot}3 \pm 0{\cdot}71$	$14{\cdot}5 \pm 0{\cdot}34$	$15{\cdot}4 \pm 0{\cdot}10$	$15{\cdot}2 \pm 0{\cdot}00$

* Biofizika **3:** No. 4, 454–458, 1958.

(2) The plants were cut on the day of irradiation. The new growth formed in the next 14 days, and in the 7 days following, was used; by this time the meristem cells had ceased to grow in all plants given doses of 0·3, 0·5 and $1·0 \times 10^4$ r.

Control	1000 r	3000 r	5000 r	10,000 r
$9·7 \pm 0·00$	$11·1 \pm 0·60$	$20·3 \pm 0·24$	$21·1 \pm 0·55$	$20·0 \pm 0·00$

FIG. 1. Type 599 winter wheat.
(1) Control, (2) 1000 r, (3) 3000 r, (4) 5000 r, (5) 10,000 r. The plants were irradiated at 4 days old and were cut back to the level of the coleoptiles immediately after irradiation. The plants are seen after growing for 6 days, by which time the meristem cells had ceased to elongate after doses of 3000, 5000 and 10,000 r.

(3) The plants were cut on the seventh day after irradiation. The new growth formed in the next 14 days and, at 7 days after the meristem cells had ceased to grow (Fig. 2), was used.

Control	1000 r	3000 r	5000 r	10,000 r
$8·6 \pm 0·15$	$10·0 \pm 0·43$	No leaves grew after cutting		

The data show that in all irradiated leaves the dry weight increased greatly. This was particularly so at the three highest doses, i.e. where the growth was completely suppressed, and where the leaves only extended because the cells did. This common feature also made the dry weights very similar.

If the leaves were kept longer in the light (second series) there was practically no effect with the control plants, but the irradiated ones showed an increase in dry weight. This was particularly so at the three highest doses, i.e. where the growth was completely suppressed. The similarity between these three sets, and the marked difference from the set irradiated at 1000 r indicated that the growth depression was the cause of the increase in dry weight.

In the third series the set irradiated at 1000 r showed only a small increase in dry weight. At the three higher doses the leaves did not elongate after complete vacuolization, and so we could not obtain any results.

A check series with certain modifications was run, using 500 r as well as the other doses; this suppressed the growth, but less than did 1000 r.

(1) The plants were cut on the day of irradiation. The new growth formed in the 5 days following was used; the meristem cells in the plants irradiated at the three highest doses had then not finished expanding.

Control	500 r	1000 r	3000 r	5000 r	10,000 r
9·6 ± 0·23	10·3 ± 0·23	11·3 ± 0·23	12·8 ± 0·23	13·1 ± 0·18	13·1 ± 0·29

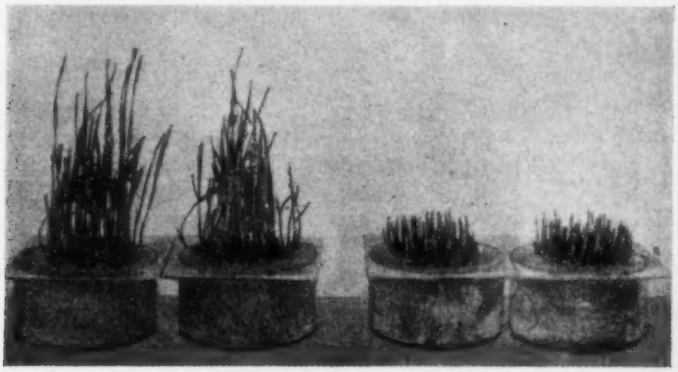

FIG. 2. Type 599 winter wheat.
(1) Control, (2) 3000 r. The plants are seen after growing for 7 days after cutting and for 14 days after irradiation. (Plants irradiated at 4 days old.)

(2) The plants were cut on the day of irradiation. The new growth formed in the 5 days following the first determination was used; the meristem cells in the plants irradiated at the three highest doses had then ceased to expand; the leaves were left a further 3 days in the light.

Control	500 r	1000 r	3000 r	5000 r	10,000 r
9·5 ± 0·00	10·25 ± 0·05	12·4 ± 0·10	16·8 ± 0·10	18·5 ± 0·05	18·9 ± 0·55

This experiment gave much the same result as the previous one, and indicated that the growth depression was the cause of the increase in dry weight. This was particularly so at the three highest doses, i.e. where the growth was completely suppressed. The dry weight increased with the time elapsed since irradiation. In the case of the 500 and 1000 r doses the dry weight increased with the dose.

DISCUSSION

In 1905 Koernicke [2] showed that X-rays caused the roots to stop growing and thicken in beans and peas, while the meristem disappeared at the root-tips. Harris *et al.* [3] have found that low X-ray doses cause the diastase activity of wheat seeds to increase, along with a slight rise in the sugar content. These observations were made on different species of plants without proper dosimetry, but they nevertheless show related effects, and demonstrate that the growth depression is the cause of the other effects.

Our results show that the growth depression produced by X-rays causes the cells to accumulate dry matter, the more so the greater the growth depression, up to the

limit where growth entirely ceases (3000 r in our experiments). However, the time the plants have stood in photosynthetically active light also has a marked effect; the dry weight increase rises with the time allowed in the light.

The physiological changes produced in wheat seedlings by irradiation may be supposed to be as follows. The primary effect is on growth, which is partially suppressed by 500 or 1000 r, and completely by 3000 r or more. The critical dose is 3000 r, and further increase in dose produces no further effect. The materials produced for growth purposes are not utilized, and therefore accumulate. In the meristem cells this produces an additional tendency to expand, which leads to vacuolization, and finally to loss of the power to grow. The increase in size is caused by elongation of the cells, and not by growth proper. This explains why root hairs are formed in the meristem zones of roots.

Growth is much more sensitive to irradiation than are other physiological processes such as synthesis and transport. Osmotic processes are very radio-resistant. Water absorption and turgor maintenance are not disturbed even by doses of 100,000 r.

SUMMARY

(1) The dry weights of type 599 winter wheat leaves irradiated at 500, 1000, 3000, 5000 and 10,000 r at 4 days old have been determined.

(2) The dry weights increased with the growth depression. Above the dose producing complete depression there was no further effect.

(3) The dry weights increased with the time the plants were allowed to stand in the light, particularly in the plants in which growth was completely suppressed.

(4) The increase is assigned to the accumulation of materials normally utilized in growth.

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DEVELOPMENT OF SOFT WHEAT SEEDLINGS FROM THERMAL-NEUTRON IRRADIATED SEEDS*

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THE optimal radiation doses and conditions required to produce mutations in agricultural plants are very important in developing selection work. It has been claimed [1, 2] that the depression of growth in seedlings and the number of chromosome

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cross-overs in the first mitoses in the rootlets serve as measures of the genetic damage. Gelin [2] has found positive correlations between the number of chromosome cross-overs in the first mitoses, the sterility of the plants from irradiated (K_1) seeds and the number of mutations in the offspring (K_2). A similar correlation with retardation in growth rate has also been found. Anoxia during irradiation, humidities of 10–18 per cent and temperatures raised to 25°C during seed ripening all reduce these effects if the radiation used is of low specific ionization [1, 3]. Hence if the number of chromosome cross-overs in the first mitoses and seedling growth are followed together we can get a useful index of the radiosensitivity and of the radiation doses producing the optimum number of mutations per unit of ability to survive.

We studied the chromosome cross-overs and seedling development in *Lutescens*-62 wheat after irradiating the air-dried resting seeds in a thermal neutron flux contaminated with γ-rays. The main features studied were: (1) the structures of the nuclei in the rootlets, (2) the growth of rootlets, coleoptiles and leaves in the first 7–10 days after irradiation, (3) the times of the first and second mitoses in control and irradiated rootlets, (4) the relation of the fraction of cells with chromosome cross-overs to radiation dose, and (5) how this latter number changed during growth.

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MATERIALS AND METHODS

Lutescens-62 seeds (42 chromosomes) were irradiated in a thermal neutron flux contaminated with γ-rays in May 1956, using a hole in the concrete shield of a reactor which gave access to the reflector. The fast neutron flux was negligible; all samples were irradiated simultaneously. The doses were varied by choice of the distance within the reflector and were measured with sodium and gold monitors placed with the samples. The radiation doses given were, in neutrons/cm^2,

(I) $1{\cdot}87\times10^{14}$;	(II) $3{\cdot}42\times10^{13}$;	(III) $1{\cdot}11\times10^{13}$;
(IV) $1{\cdot}80\times10^{12}$;	(V) $4{\cdot}96\times10^{11}$;	(VI) $1{\cdot}51\times10^{11}$;
(VII) $2{\cdot}56\times10^{10}$;	(VIII) $8{\cdot}6\times10^{9}$;	(IX) $3{\cdot}71\times10^{9}$;

The seeds were then grown in petri dishes at 20–25°C. The roots were fixed in Carnoy's mixture, or in a mixture of three parts of alcohol with one of acetic acid for 3–4 hr when 0·2–1·0 cm long. After washing in 70° alcohol they were stained in acetocarmine for 30 min, then boiled for 1–2 min, transferred to a slide and pressed under a cover-slip. The preparations were examined for late anaphases and telophases showing chromosome bridges or breaks. These temporary preparations were replaced by permanent ones if the cells or resting nuclei had to be counted. For this purpose, sections 14μ thick were cut and stained with gentian violet or methyl green-pyronin.

In the growth studies the seedlings were grown on perforated plastic plates, with the roots entering tap-water or Mayer's solution. The lengths of the coleoptiles, leaves and roots were measured every day. The seedlings were kept in a case illuminated by "daylight" lamps at a temperature of about 25°C.

RESULTS

1. *Seeds*

Developing seeds are more radiosensitive, but air-dried resting seeds are more convenient to work with; a particular advantage of dry seeds is that the cells are all

the same in the germ. Cells vary in radiosensitivity during the division cycle; the rootlet cells are all at the same stage, and although one frequently sees clumps of thin Feulgen-positive filaments reminiscent of early prophase the chromosomes have not yet split up into chromatids. This is indicated by the types of chromosome cross-over observed (see below). Control seeds, and irradiated batches Nos. (I), (II), (VI) and (IX) were used. The rootlet is a multicellular formation in the germ. A longitudinal section showed some 1050 cells, and in all there must have been some 7000, all of which appeared to be in the same state. As any metabolic processes in seeds are very slow, the conditions of all cells are very similar, which was very important in our work, since water content, partial pressure of oxygen, and certain other factors, greatly influence the radiosensitivity.

Before growth began no difference could be detected between the control seed and ones given very large doses (I and II). The methyl green–pyronin stained the rootlet cytoplasm a strong pyronin colour (reddish) but the nucleoli only weakly, so the nucleoli could not be counted at this stage. Granules stained by the methyl green typical of resting nuclei were seen. In the middle the nuclei were round or oval, but at the edges they were somewhat flattened, like the cells. The cytoplasm at the growth point was also intensely stained by the pyronin. In other tissues the cytoplasm stained a light rose, with the nuclei staining as in the rootlet.

Marked differences could be seen between the control and irradiated seeds at 10, 17 and 35 hr after germination. The irradiated nuclei contained more nucleoli, and since the nuclear RNA accumulates in the nucleolus, we decided to determine whether the nucleoli actually contained more total RNA, since ionizing radiations greatly affect nucleic acid metabolism. The increase in the number of apparent nucleoli might be due in fact to chromosome fragments. No mitoses were seen in either control or irradiated seeds fixed at 10 hr. At 17 hr a few mitoses were seen in group (IX), but none in any of the others. The coleorhyzis had appeared in the controls, and in about 50 per cent of cases in groups (VI) and (IX), but none at all in groups (I) and (II). Fixation at 35 hr revealed many mitoses in the control and group (VI). The results for 17 hr fixation are the most interesting in relation to the nucleoli, since no mitoses were seen in the control at this time, and mitosis probably affects the nucleoli greatly. Table 1 gives the nucleoli counts for controls and seeds receiving the largest doses.

The nuclei receiving high doses show more nucleoli even at 10 hr, but the result is not statistically reliable. The excess at 17 hr is quite reliable. To determine whether the total mass of the nucleoli also increased we sketched the nucleoli in 100 nuclei

Table 1. Nucleoli counts for nuclei fixed 17 hr after germination

Group No.	No. of nucleoli				Total No. of nuclei	M	σ	M_{diff}
	1	2	3	4				
Control	59	125	28	0	212	$1{\cdot}86 \pm 0{\cdot}04$	0·63	
I	29	79	93	22	223	$2{\cdot}48 \pm 0{\cdot}055$	0·84	$M_I - M_c = 0{\cdot}62 \pm 0{\cdot}07$
II	34	75	83	12	204	$2{\cdot}36 \pm 0{\cdot}05$	0·85	$M_{II} - M_c = 0{\cdot}5 \pm 0{\cdot}067$

using a projection fitting, with group (I) and control seeds fixed 17 hr after germination. One hundred control cells contained 159 nucleoli of total mass 263 mg, while in group (I) 100 cells contained 185 nucleoli of weight 329 mg. The ratio of areas of the nucleoli was 1·25. Calculated as volumes (taking the nucleoli as spherical) gave the ratio of the totals as about the same. Hence the total mass of the nucleoli is increased by large radiation doses.

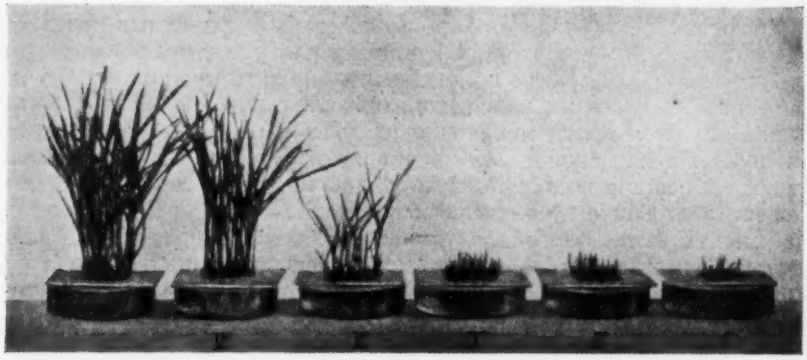

FIG. 1. Seedlings from *Lutescens*-62 wheat 12 days after germination. The control is on the left; the numbers are those of the groups.

2. *Seedlings*

Growth retardation increases with dose; this is very clearly seen with groups (I), (II) and (IV) (Fig. 1), in which growth ceased completely or the seedlings died in many cases. The roots were covered with root hairs right down to the ends. In groups (IV) and (V) the roots sometimes forked and shortened before the seedlings died. A few seedlings continued to grow and developed leaves in group (V). The germination percentages and rates were only appreciably reduced in groups (I) and (II); in groups (III–IX) the values were even slightly higher than for the control.

3. *Cytological analysis of the rootlets*

A. The first mitoses. As the chromosome cross-overs were counted in the first mitosis we had to establish the maximum rootlet length which gave adequate late anaphases and telophases, but no second mitoses. It was found that any length from 2 to 8 mm was suitable; above 8 mm second mitoses can occur. At 9–10 mm we usually found micronuclei, which form from fragments and lack centromeres, and which stop at the first mitosis. In groups (I)–(III) there were no mitoses at all, although the roots grew to lengths of 5–8·5 mm before dying. Froier and Gustafsson [4] stated in 1941 that cellular growth and mitosis are partially independent, and that vital processes continue in the cytoplasm even when the nucleus is destroyed. Ehrenberg and Nybom [3] have shown that neutron-irradiated seedlings can grow larger by cellular expansion. Many cells with micronuclei were seen in the very short (2–3 mm) rootlets produced in groups (V) and (VI). Since these micronuclei arise from fragments which have not moved over to the poles during mitosis, we must suppose that the mitoses take place very early. It was found that mitosis actually

occurred in rootlets 1 mm long in these groups. The nuclei did not fragment during interphase even at the highest doses (groups (I)–(III)).

B. Chromosome cross-overs in rootlets. These cross-overs were examined on the temporary acetocarmine preparations. The late anaphases and telophases with bridges and breaks were counted. All cross-overs were of chromosome type (double bridges and fragments). No chromatid bridges or fragments were found. Table 2 gives the results; it was also found that the number of damaged cells was positively correlated with the stunting. In groups (I)–(III) (no mitoses) and (IV) (100 per cent of dividing cells showing cross-overs) growth ceased at the coleoptile stage. In (V)

TABLE 2. ANAPHASE AND TELOPHASE CELLS SHOWING CHROMOSOME CROSS-OVERS, AND ROOTLET GROWTH

Group No.	Total dose (n/cm^2)	No. of roots used	Nos. of anaphases and telephones		Nos. of anaphases and telophases showing cross-overs (%)	Mean rootlet length 6 days after germination (mm)
			Studied	Showing cross-overs		
IX	$3{\cdot}71\times10^{9}$	19	678	13	$1{\cdot}9\pm0{\cdot}27$	51·0
VIII	$8{\cdot}6\times10^{9}$	16	850	94	$11{\cdot}06\pm1{\cdot}07$	36·0
VII	$2{\cdot}56\times10^{10}$	14	539	117	$21{\cdot}7\pm1{\cdot}78$	32·1
VI	$1{\cdot}51\times10^{11}$	14	668	259	$38{\cdot}8\pm1{\cdot}91$	28·3
V	$4{\cdot}96\times10^{11}$	10	339	275	$81{\cdot}1\pm2{\cdot}1$	21·4
IV	$1{\cdot}80\times10^{12}$	7	201	201	100	15·3
III	$1{\cdot}11\times10^{13}$	5	No mitoses			6·0
II	$3{\cdot}42\times10^{13}$	11				3·8
I	$1{\cdot}87\times10^{14}$	4				1·1

(19 per cent free from aberrations) some of the plants (10–15 per cent) formed leaves, and some of these set seeds under field conditions. The other groups grew more or less normally, although group (VI) lagged behind the control.

We also studied the fate of the aberrant cells during subsequent rootlet growth in a preliminary way by counting the anaphases with chromosome breaks in group (VI) seedlings, which had roots 9 cm long at 10 days old, and compared the result with the number of aberrations in the first mitoses. Only bridges were seen; these bridges, seen in the later mitoses (not in the first), form from dicentric chromosomes which have moved as a whole from both centromeres to one pole in previous mitoses. These 9 cm roots showed rather fewer bridges than at earlier stages, namely 110 in 521 anaphases or telophases, or $21{\cdot}1\pm1{\cdot}75$ per cent, as against $38{\cdot}8\pm1{\cdot}91$ in the first mitoses.

DISCUSSION

Our results clearly show that nuclear damage plays a major or even decisive rôle in seedling stunting consequent on seed irradiation, since there is a clear positive correlation between stunting and number of cells with damaged nuclei. There was also a critical degree of damage below which growth and development proceeded. The highest doses (groups (I)–(III)) produced the most damage, but no changes in the cells could be seen which might indicate that mitosis was beginning; only the number of nucleoli increased in certain cases, accompanied by a general increase in

nucleolar mass. Nucleic acid metabolism is clearly greatly disturbed, and RNA accumulates in the nucleoli, which prevents mitosis beginning. A more detailed study is required; in particular, it is important to determine whether the increase in nucleolar mass arises from RNA accumulation or vacuolization. The oxidation processes should also be studied, since it has been stated [5] that cells need energy in order to begin to divide, and that the most radiosensitive processes are DNA synthesis and energy production, which can be affected independently. It is at present unclear whether chromosome damage, interference with DNA synthesis or disturbance to energy production prevent nuclei dividing at high radiation doses.

At a lower degree of damage (group (IV)) a few mitoses were seen, but all were abnormal, as either fragments or bridges were present, to the extent of 5·6 aberrations per mitosis. Many metaphases never go over to anaphases; if 100 per cent of the dividing cells are damaged, or if there are no mitoses, the seedlings stop growing at the coleoptile stage, and the cells thereafter merely enlarge. Very many more mitoses were found in group (V), with chromosome faults in $81 \pm 2{\cdot}1$ per cent of cells. The growth was very stunted, but some plants grew and even gave seeds. In group (VI) only $38{\cdot}3 \pm 1{\cdot}91$ per cent showed chromosome damage, and development was normal although somewhat retarded.

The cells died at the interphase stage in groups (I)–(III). Our data confirm Howard's [6] opinion that two types of cell death occur, namely (1) after the first mitosis because the chromosome balance is disturbed, and (2) at interphase. The first type is responsible for cell death at the lower doses.

Gray and Scholes [7] have studied the fate of micronuclei, cross-overs and other types of damage using broad bean (*Vicia faba*) roots acted on by various types of ionizing radiation. They also found that the number of damaged nuclei decreased as the cells grew. The micronuclei were most numerous at 2 days after irradiation, thereafter falling off gradually to day 10; five mitotic cycles occur in this time. The morphological anomalies were most marked at day 6. By day 10 cell growth was approaching normal. The fates of dicentric chromosomes and of cells with or without disturbed chromosome balances demand a special study.

SUMMARY

(1) Nuclear damage caused by ionizing radiation plays a decisive part in retarding growth in seedlings.

(2) Cells die at two stages, namely early, at interphase, as mitosis begins (groups (I)–(III)) and late, due to disturbances in the chromosome balance (group (V), and at lower doses).

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THE ACTION OF IONIZING RADIATION ON CELL DIVISION: AN INVESTIGATION OF LOCAL AND REMOTE EFFECTS*

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IT is the accepted belief that the effect of radiation is brought about both by local influences (as a result of a direct or indirect action), and by the reflection of distant damage. However, our knowledge of the mechanism of development of the radiation syndrome is at present confined to statements that such activity is possible.

The reflected influences, according to the findings of various workers, produce different changes: a leucopoenic reaction [1–5], suppression of RNA and especially DNA synthesis [6–18], a fall in the blood pressure, suppression of cell-division [13, 14] and even death of cells in animals [15–17].

Our ideas on the mechanism of transmission of these distant effects are not clear. The most widely held view is the hypothesis of circulation of radiotoxins in the blood, leading to the appearance of the changes listed above. It should be mentioned, however, that analysis of the material facts obtained in a number of investigations already cited and of other evidence in the literature does not, in our opinion, support the conclusions of these authors. We will mention only a few objections.

In experiments on parabiosis [19], morphological descriptions given without numerical details of the results, cannot serve as valid arguments in support of or against the existence of radiotoxins. The presence of a leucopoenic effect during irradiation of a skin flap [20] cannot be regarded as evidence on account of the small amount of material and the lack of any suitable statistical treatment. It must also be pointed out that irradiation of the hind limbs of a mouse, thereby irradiating areas of skin at least equal in area to those in Kuznetsova's experiments [21], does not have any leucopoenic effect [22]. Destruction of cells, also connected by certain workers with the distant action of radiation, has been convincingly shown by Barakina [23] and Kohn [24] to be due to the local action of radiation. The suppression of growth of *B. coli* by the action of serum from irradiated animals [13] is in contrast to experiments in which a fall in the bactericidal properties of the blood during radiation sickness was shown [25]. The existence of certain distant effects or of the humoral mechanism of their development are thus not yet adequately proved.

In the present paper we give the results of experiments in which the local and distant effects of radiation on the process of cell division are analysed, and the mechanism of transmission of the reflected effects is studied. The experiments were performed on white mice. As test objects for studying the action of radiation on the

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process of cell division we used corneal epithelium, in which the normal mitotic index is sufficiently high (approximately 1 per cent). In order to prevent the effect of daily variations in mitotic activity on the experimental results, the animals were always killed at 10 a.m., except in the case of experiments in which the interval of time after irradiation was 6 hr, when they were killed at 5–6 p.m. The enucleated eyes were fixed in Bouin's or Navashin–Krylov's fluid, and then total corneal preparations were made and stained with haematoxylin by Caracci's or Ehrlich's method or with haemalum. In each animal the number of mitoses in both corneas was counted (100 fields in each). One field of vision contained $203 \pm 1{\cdot}9$ cells, i.e. in both corneas, in order to determine the number of mitoses in various phases, about 40,000 cells had to be

TABLE 1. DISTRIBUTION OF PHASES OF MITOSIS IN THE CORNEAL EPITHELIUM (IN 100 FIELDS OF VISION) IN CONTROL MICE (IN PER CENT)

Time of death	Form of experiment	No. of animals	P	M	A	T	Total No. of mitoses
10 a.m.	Parabionts	17	$44{\cdot}9 \pm 2{\cdot}6$	$31{\cdot}6 \pm 2{\cdot}6$	$7 \pm 0{\cdot}7$	$16{\cdot}5 \pm 0{\cdot}6$	$228{\cdot}2 \pm 13{\cdot}8$
	Singles	24	$34{\cdot}3 \pm 1$	$39{\cdot}7 \pm 2{\cdot}7$	$8{\cdot}2 \pm 0{\cdot}2$	$17{\cdot}8 \pm 1{\cdot}2$	$232 \pm 7{\cdot}7$
5–6 p.m.	Parabionts	6	$37{\cdot}1 \pm 4{\cdot}5$	$31{\cdot}7 \pm 3{\cdot}2$	$13{\cdot}6 \pm 1{\cdot}8$	$17{\cdot}6 \pm 1{\cdot}2$	$118{\cdot}7 \pm 21$
	Singles	19	$34{\cdot}8 \pm 1{\cdot}9$	$37 \pm 10{\cdot}5$	$10 \pm 0{\cdot}8$	$18{\cdot}2 \pm 1{\cdot}1$	$106 \pm 10{\cdot}2$

Note. P, M, A and T indicate the pro-, meta-, ana- and telophase respectively. In all cases the arithmetic mean values are given, with the mean error.

counted. The results of these counts were averaged and from the values obtained the mitotic activity of the corneal epithelium in the particular animal was judged. The number of abnormally dividing cells, including all cells with chromosomal aberration, was determined in counts of 200 anaphases. In all the experiments given below the animals were irradiated with X-rays, with a dose of 700 r (190 kV, 15 mA, filtered by 0·5 mm Cu + 0·75 mm Al, dose rate 80–100 r/min).

In the first series, total irradiation of the animals was performed; in the second the eye was locally irradiated, the body of the animal being screened by a lead plate. In the third series of experiments the head was screened and the rest of the body irradiated; in the fourth the head and adrenals were screened. The latter were covered by a plate measuring $4 \times 1{\cdot}6$ cm, placed lateral to the spine at the costal margin. In the fifth series the head and pelvis of the animals were screened, for which we used a lead plate like that used for protecting the adrenals.

In the sixth and seventh series on inbred mice of strains A and C_{57} (black),* the experiments were carried out using parabiosis. For the experiments we selected pairs of the same weight, sex and litter. The operation of parabiosis was performed by the method of Bunster and Meyer [26] 8–10 days before irradiation of one parabiont. The right partner in the sixth series was irradiated totally, and in the seventh series, the head of the animal was screened. In all the series (2–7) of experiments the screen used was a lead plate 3 mm thick. Measurements with a condenser dosemeter showed that less than 1 per cent of the radiation passed through the screen. The animals in the first six series were decapitated after 6 hr and 1, 3 and 5 days, and in the seventh

* The pure strains of mice were obtained from the Institute of Genetics of the Academy of Sciences of the U.S.S.R.

series after 1 and 3 days following irradiation. Simultaneously the mitotic activity of single and parabiont control mice was investigated. The operation of parabiosis in the control group was performed 8–10 days before the animals were killed. From Table 1 it can be seen that the number of mitoses in the single and parabiotic animals was the same.

There are reports in the literature [27] of certain differences in radio-sensitivity between mice of strains A and C_{57} (black). From a study of the mitotic activity of the control parabiont mice, and also from its change after irradiation in our experiments carried out on mice of both strains, we were unable to discover any difference of this sort. For this reason the respective findings were combined in the Tables.

A comparison of the results of total irradiation of the animals and local irradiation of the eyes shows a great similarity between the changes in all three criteria studied.

(1) The mitotic activity in both series fell to zero in the first 6 hr and then gradually recovered, so that after 5 days it was still 1·5–2 times below the normal level (Table 2).

TABLE 2. MITOTIC ACTIVITY IN SINGLE MICE AFTER IRRADIATION WITH 700 r (IN 100 FIELDS OF VISION)

Series	After 6 hr	After 24 hr	After 3 days	After 5 days
1	0(7)*	42 ± 7·4(6)	87 ± 10·7(10)	151 ± 12·3(7)
2	0(7)	41 ± 9·1(5)	102 ± 7·1(10)	121 ± 9(12)
3	119·6 ± 8·2(8)	85·8 ± 16·2(8)	79·2 ± 14·1(9)	194 ± 19·9(9)
4	135 ± 16·8(9)	132 ± 5·1(11)	122 ± 9·9(11)	183·7 ± 10(10)
5	79·9 ± 8(8)	89 ± 14(6)	153 ± 8·2(7)	179·2 ± 22·9(5)

* The figures in brackets indicate the number of animals examined.

(2) The relative duration of the different phases of mitosis was hardly affected by ionizing radiation, as shown by analysis of the percentage distribution of the phases of mitosis. It can be seen from Table 3 that with total and local irradiation the percentage distribution of phases of mitosis agreed well with the normal values. In the experiments of the first series, only after a period of 5 days was the number of prophases diminished by 5·9 per cent ($P < 0·001$) and the number of ana- and telophases by 4·3 per cent ($P < 0·01$) and 4·6 per cent ($P < 0·01$) respectively, which is possibly connected with division of cells whose chromosome apparatus is profoundly disturbed.

(3) The number of pathological mitoses also does not differ appreciably after total and local irradiation, with the exception of the experiments lasting 5 days. In these experiments, during total irradiation there were 15 per cent more anomalous mitoses ($P < 0·05$). The well-marked agreement between the results of total and local irradiation therefore enables us to conclude that the above-mentioned changes are due to the local action of radiation. However a number of firmly established facts concerning the neurohumoral regulation of normal cellular division, and the admitted possibility of production of radiotoxins, suggested that radiation may also exert a distant effect on the process of cell division.

In the experiments of the third to fifth series it was shown reliably that the mitotic activity of cells may be considerably suppressed also by the distant effects of radiation.

However, these changes differ in nature from those observed during the local action of X-rays.

(1) In distant as opposed to local activity the suppression of mitotic activity by the same dose of radiation was less pronounced, appeared later (24 hr after irradiation) and stayed at the same level for 3 days, after which it rapidly returned to values close to normal (see Table 2). The relative duration of the phases of division in these experiments, as also in the case of local activity, differed only slightly from normal (see Table 3).

(2) The distant effect did not cause the appearance of cells with chromosomal aberrations, which usually appeared as a result of the action of radiation.

TABLE 3. DISTRIBUTION OF PHASES OF DIVISION OF THE CORNEAL EPITHELIUM AFTER IRRADIATION WITH 700 r (IN PER CENT)

Series	After 6 hr				After 24 hr			
	P	M	A	T	P	M	A	T
1	0	0	0	0	38±5·3	35±3·2	8·4±0·9	18·6±2·8
2	0	0	0	0	35·7±1·2	39·9±2·6	7·3±0·6	17·2±2·7
3	27·4±5	39·4±2·3	11·6±0·9	21·6±3·1	26·8±2·9	38±2·7	11·2±1	24±3·5
4	36·1±3·5	38±1·6	10·4±1·3	15·5±1·9	39·2±3·4	38·5±2·4	7·2±0·8	15·1±1·3
5	30·5±4·2	36·5±2·2	13·5±0·9	19·5±2·8	35·2±1·5	34·5±1·1	9±1	21·3±1·9
	After 3 days				After 5 days			
1	45·5±2·3	32·0±3·2	8·8±1	13·7±1·9	28·4±1·6	36·7±1·3	12·5±0·8	22·4±0·8
2	37·1±3·8	36·2±1·2	8·6±0·9	18·1±1·2	35·6±2	36·3±1·5	9·2±0·5	18·9±0·9
3	40·7±4·6	32±2·7	8·7±1·7	18·6±2·6	35·2±3·2	37·3±2·8	9·8±0·8	17·7±0·5
4	35·1±3·5	39±3·4	8·6±0·5	17·3±1·2	30·6±3·8	42·4±2·5	8·4±0·7	18·6±1·1
5	33·7±1·3	39·9±1·1	9·4±0·6	17±1·3	27·6±2·8	40·2±3·3	12·6±0·7	19·6±1

(3) The results of the first and second series showed that the irradiated animals themselves do not influence greatly the changes in cell division arising as a result of the local action of ionizing radiation on this process. During distant action, the volume (and probably the site) of the part of the body affected play a definite rôle. It can be seen from Table 2 that additional screening (except of the head) of a comparatively large area of the body, including the region of the adrenals (fourth series), led to a slight, significant ($P<0·05$) increase (roughly by 50 per cent) in the total number of mitoses 1 and 3 days after irradiation. This increase in the number of dividing cells is evidently not connected with the screening of the adrenals themselves, as is shown by the results of the control (fifth) series of experiments in which, in addition to the head, the pelvis was screened as well. It was found that the mitotic activity in this series as a whole did not differ from that in the fourth series. However, this is not to deny that the pituitary–adrenal system may not play some part in the development of certain signs of the reaction to radiation, as has often been pointed out in the literature [28–31]. It may be thought that of more essential importance in the reaction of the pituitary–adrenal system would be those changes which develop in the body, rather than those arising in the effector organ—the adrenals—by the direct action of radiation on them.

The special features of the action of radiation on cell division are shown in Table 4.

It is not yet clear why the distant effects of radiation do not appear during total irradiation. One would think that if indirect effects take place, total irradiation of animals must result in more severe changes than those following local action on the corneal epithelium. Possibly in comparison with the distant effect, the local action of X-rays causes such profound changes in the cell that any form of additional activity cannot produce any further changes.

In the next two series of experiments an attempt was made to study in what way the distant effect of radiation is carried out. A very suitable subject for solving this particular problem is animals joined together in parabiosis. As Van Dyke and his co-workers [31–32] showed, an exchange of blood is established between parabionts (in their experiments they used rats) within 3–4 days of operation. By means of erythrocytes labelled with ^{59}Fe these workers were able to show that in 24 hr there is a ten-fold interchange of blood between the parabionts. Repeating Van Dyke's experiments we also were satisfied that 4–5 days after operation, red blood cells labelled with ^{32}P, injected into one partner, could be found in the blood of the other partner after a few minutes.

TABLE 4. COMPARATIVE FEATURES OF THE LOCAL AND DISTANT ACTION OF RADIATION ON CELL DIVISION IN THE CORNEAL EPITHELIUM

Criterion	Local action	Distant action
Mitotic activity	Sharp depression within a few hours of irradiation, slow recovery	Depression is less pronounced and later to appear (>24 hr), rapid recovery
Chromosomal disturbances	Always present	Not present
Relation to dimensions of part of body irradiated	No	Yes

In the sixth series of experiments, during total irradiation of the right parabiont, its completely screened partner showed changes in mitotic activity which were very similar in their development but only half as pronounced as those which were observed in experiments in which the head of single animals was screened (Table 5). The

TABLE 5. MITOTIC ACTIVITY IN PARABIONT MICE AFTER IRRADIATION WITH 700 r (IN 100 FIELDS OF VISION)

Series	After 6 hr	After 14 hr	Afteer 3 days	After 5 days
6a	0(5)*	36·3±3·7(7)	82·7±14·4(9)	126·2±19·3(4)
6b	97·2±19·3	175·8±20·3†	158·7±22·8	223·5±33
7a	—	162·5±15·4(8)	152·2±14·6(5)	—
7b	—	175·8±20·3†	156·4±24·4	—

* The figures in brackets indicate the number of animals examined. 6a—total irradiation of the parabiont; 6b—screened parabiont; 7a—irradiation of parabiont with screening of the head; 7b—screened parabiont.

† These values were obtained by combining the results of the experiments.

relative duration of the phases of mitosis in these experiments was not altered (Table 6), except in experiments lasting 3 days, in which a fall in the number of prophases by 9·7 per cent ($P<0{\cdot}01$) was observed. No cells with chromosomal aberration were found. The changes in mitotic activity of the irradiated partner and also the number of anomalously dividing cells, were in good agreement with these values in single animals totally irradiated with the same dose (Fig. 1, Table 5). As regards the relative duration of the phases of division, no significant differences from the control parabiont mice could be detected (see Table 6).

These differences in mitotic activity in the screened parabiont could be explained by the halving of the concentration of the factors which suppress cell division on,

TABLE 6. DISTRIBUTION OF THE PHASES OF DIVISION OF THE CORNEAL EPITHELIUM IN PARABIONTS (IN PER CENT)

Series	After 6 hr				After 24 hr			
	P	M	A	T	P	M	A	T
6a	0	0	0	0	51·1±6	27·1±3·2	7·5±1	14·3±3·2
6b	43±4	32·8±4·5	8·1±1·1	16·1±2·4	43·9±22·8	31±2·6	9·4±1	15·7±0·9
7a	—	—	—	—	44·1±4	32·7±3	8·8±1	14·4±1·3
7b	—	—	—	—	43·9±2·8	31±2·6	9·4±1	15·7±0·9
	After 3 days				After 5 days			
6a	41·8±2	34·3±1·8	8·3±1·4	15·6±2·4	31·4±5·2	39·1±5·9	11·8±1	17·7±1·2
6b	35·2±2·8	37·6±3·5	9±0·7	18·2±2·7	39·2±3·2	31·5±5·1	10·3±0·9	19±1·2
7a	33·5±2·7	37·8±4·2	10·8±1	17·9±2·1	—	—	—	—
7b	36·5±2·7	37·1±3·7	11·5±1	14·9±1·5	—	—	—	—

Note. 6a—total irradiation of parabiont; 6b—screened parabiont; 7a—irradiation of parabiont with screening of the head; 7b—screened parabiont.

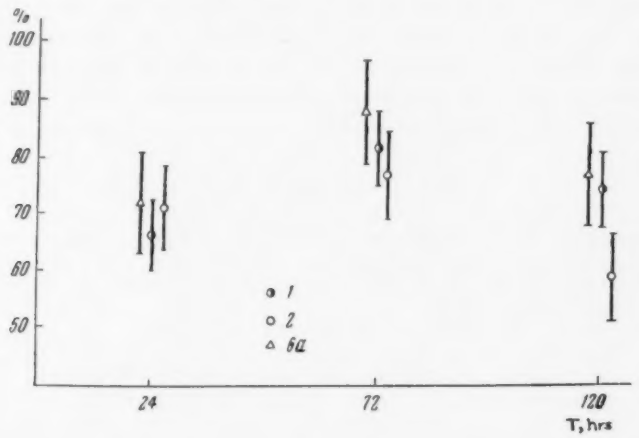

FIG. 1. Number of pathological mitoses (per 200 anaphases) in the corneal epithelium of mice after irradiation with 700 r (in per cent). 1—total irradiation; 2—local irradiation of the eyes; 6a—total irradiation of a parabiont; T—time after irradiation.

account of their dilution in the blood of the two parabiotic animals. In support of this view are the results of the seventh series of experiments, in which the head of the irradiated partner was protected. It was found that in this form of the experiment the mitotic activity of the corneal epithelium of both parabionts was the same and was twice as high as in single animals with screening of the head (see Table 5). The percentage distribution of phases of mitosis in this series of experiments was only slightly different from the control. Only in experiments lasting 3 days was there observed in both partners a diminution in the prophases by 11·4 per cent ($P<0{\cdot}01$) and 8·4 per cent ($P<0{\cdot}05$) and an increase in the anaphases by 3·8 per cent ($P<0{\cdot}01$) and 4·5 per cent ($P<0{\cdot}01$) respectively.

On the basis of experiments on parabiotically joined animals it may be concluded that the blood of irradiated animals contains certain factors which have the power to reduce temporarily the mitotic activity of the cells. The mechanism of transmission of the distant effect on to the mitotic activity of the corneal epithelium is essentially humoral. It may be thought that these hypothetical substances are possibly radiotoxins, and the observed effects could then be connected with the increase or decrease in the amount of the substances which are normally present in the blood, influencing mitosis (for example, hormones), or with changes in their proportions.

SUMMARY

(1) X-rays have both a local and distant action on the process of cell division. The local effect appears as: (1) a sharp fall in the number of mitoses in the first hours after irradiation; (2) the appearance of cells with chromosomal aberration; (3) the absence of relationship between the effect of radiation and the dimensions of the area of the body irradiated at one time. The distant action: (1) leads to a less pronounced depression of mitotic activity which appears later; (2) does not cause the appearance of chromosomal aberration; (3) has an effect which depends on the volume of body tissue irradiated.

(2) In experiments on parabiotically joined mice it has been shown that the distant action of radiation on cell division is effected by means of factors which circulate in the blood. It was found that the halving of the concentration of the mitosis-suppressing factors in the blood of the parabionts leads to a reduction by approximately one half in the distant effect.

Translated by B. HAIGH

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A COMPARISON OF THE EFFECTS OF α- AND β-RAYS ON MICRO-ORGANISMS*

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THE detailed distribution of ionization and excitation plays an important part in determining the biological effects of radiation; radiations giving differing ionization and excitation densities along their tracks differ in biological effect. When α-particles act on viruses, bacteriophages, small bacteria and some enzymes the ion yield is appreciably lower than with X-rays or other radiation giving low-density tracks [1–4]. This is only found for single-hit effects, since if one or two ionizations suffice to produce the effect, more are superfluous and are wasted, and so the yield per ionization unit is reduced. However, in most cases the higher-density radiations produce the greater effect. Hollaender [5] has shown that α-particles and protons are much more lethal to dry *Aspergillus terreus* spores than are X-rays. Similar results have been found for moulds [6].

Gray [8] has shown that fast electrons produce 55 per cent of the chromosome damage of α-particles in aerobic conditions, and 2 per cent in anaerobic ones. The efficiency in relation to chromatin filaments is only 1 in 15,000 of that for α-particles; similar data on the high relative effectiveness of α-particles have been given by other

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workers [1, 8, 9]. Differences between radiations are even found with pure water; with X-rays the H_2O_2 formed depends on the pH, on the temperature and on the dissolved oxygen content, but with α-particles the yield is constant [10].

Since the linear ionization density depends greatly on the energy the biological effects depend on the energy as well as the nature of the radiation. When *B. coli* and *Pseudomonas aeruginosa* are subjected to X-rays of energies from 4 keV to 2 MeV the LD_{50} is 250 r at 4 keV, and 1500 r at 2 MeV [11]. The soft X-rays, which give the higher ionization density, are thus the more damaging. The same applies to bacteriophages [12].

This paper relates to a comparison of the effects of α- and β-particles on nitrogen-fixing bacteria (*Azotobacter* and root-nodule bacteria). The emitters were incorporated in the liquid or agar media. A solution of ^{235}U fission fragments (one year old) was used as the β-source; the main components were the β-emitters ^{89}Sr, ^{90}Sr, ^{90}Y, ^{95}Nb, ^{95}Zr, ^{106}Ru, ^{137}Cs and ^{144}Ce. The α-emitters used were Ra and ^{210}Po. The concentrations used were such as to produce identical α- and β-ray doses.

The parameters studied were the numbers of colonies or cells growing, the weights produced and (with the nitrogen-fixing bacteria) the sucrose uptake and nitrogen fixed. The cell sizes were also measured in certain cases. The first tests with Ra in agar containing root-nodule bacteria showed that at concentrations of from 0·05 to 50 μc/l. the growth was greatly depressed, whereas the same doses from β-emitters (corresponding to concentrations of 1–1000 μc/l.) produced no effect, or even a slight stimulation. These amounts of Ra produced a considerable increase in cell size, and numerous giant cells. Prolonged growth under these conditions caused the cultures

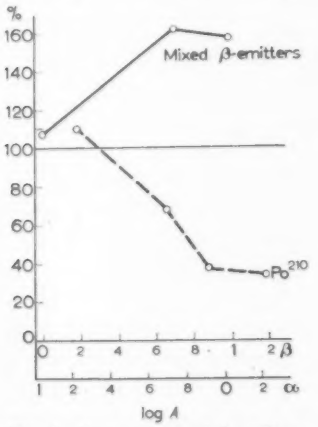

FIG. 1. Comparison of the effects of ^{210}Po α-rays and mixed β-rays (^{235}U fission fragments) on root-nodule bacteria at concentrations (mc/l.) giving approximately equivalent doses. Deviation from the dry weight of the control for liquid cultures on the first to third day of culturing (in percentage as mean of all experiments).

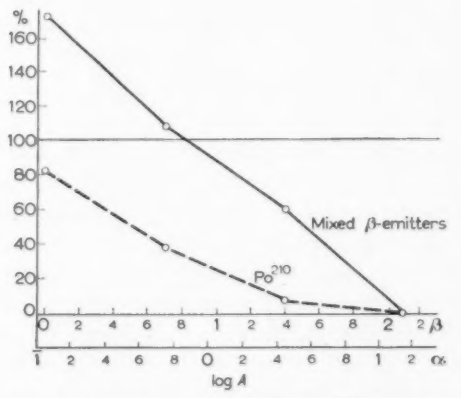

FIG. 2. Comparison of the effects of ^{210}Po α-rays and mixed β-rays (^{235}U fission fragments) on *Azotobacter*, at concentrations (mc/l). giving approximately equivalent doses. Deviation from the dry weight on the control for agar cultured in the first 3 days of culturing (in percentage, as mean of all experiments).

TABLE 1. DRY WEIGHT OF ROOT-NODULE BACTERIA PER 100 ml FOR CULTURES RECEIVING EQUIVALENT β-RAY AND ^{210}Po α-RAY DOSES

Days of culturing	Control	β-emitters (^{235}U fission fragments) 5 mc/l.		α-emitter (^{210}Po), 0·8 mc/l.	
	Weight (g)	Weight (g)	% of Control	Weight (g)	% of Control
1	0·0158	0·0154	97	0·0056	35
3	0·0248	0·0269	108	0·0078	31
9	0·0381	0·0458	120	0·0102	27
16	0·0644	0·0666	103	0·0090	14

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to recover gradually, however; after a week the number of cells and the total cell weight began to exceed the control.

The effect appears even more clearly when the actions of the β-ray and ^{210}Po α-ray doses are compared. Figs. 1 and 2 give the mean results from several experiments, and show that ^{210}Po is much more effective at concentrations equivalent in dose terms to those of the β-emitters; the same applies on long culturing (up to 21 days) under the same conditions (Table 1). The mixed α and β doses (radium) always gave effects intermediate between those for pure α- and β- emitters (Table 2 and Fig. 3).

The cultures were sometimes temporarily stimulated by α-emitters (Table 3), as occurs with β-emitters [13], but the effect was usually less marked, and lasted less long. Both α- and β-emitters had greater effects with agar media than with liquid ones.

In the experiments with *Azotobacter* the number of cells and the total cell weights were determined as well as the uptake of sugars and the nitrogen fixed. As had previously been shown for β-rays and external γ-rays [14, 15], the nitrogen fixation processes were found to be much more resistant to irradiation than cell division and growth, but all the same, the sensitivity to α-emitters was higher than for equivalent β-ray doses (Tables 4 and 5). The mixed β-emitters at 5, 25 and 125 mc/l., and Po

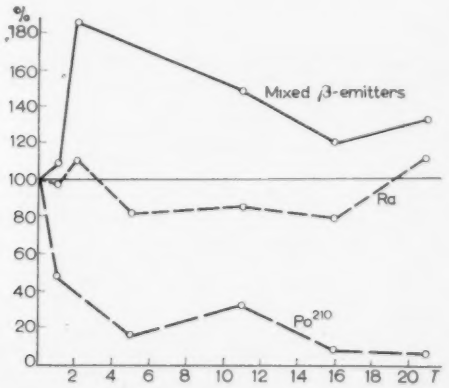

FIG. 3. Deviation from the dry weight of the control for cultures of root-nodule bacteria (in percentage), at contents of mixed β-emitters, ^{210}Po and Ra giving approximately equivalent doses. T = time of culturing, in days.

at 0·6, 3 and 14 mc/l., were approximately equivalent. Polonium has no chemical toxic effect here because even at 14 mc/l. the concentration was 3·1 μg/l. and less in the other cases.

TABLE 2. DRY WEIGHT OF ROOT-NODULE BACTERIA PER 100 ml FOR CULTURES RECEIVING EQUIVALENT MIXED β-RAY AND Ra OR ^{210}Po α-RAY DOSES

Days of culturing	Control	β-emitters (^{235}U fission fragments) 5 mc/l.		Mixed Ra α- and β-emitters at 0·25 mc/l.		α-emitter (^{210}Po), 0·8 mc/l.	
	Weight (g)	Weight (g)	% of control	Weight (g)	% of control	Weight (g)	% of control
1	0·0168	0·0182	108	0·0164	98	0·0080	48
2	0·0238	0·0440	185	0·0262	110	—	—
5	0·0468	—	—	0·0398	82	0·0080	16
11	0·0892	0·1329	149	0·0756	85	—	—
16	0·1096	0·1316	120	0·0864	79	0·0084	7·7
21	0·1638	0·2186	133	0·1848	133	0·0108	6·6

TABLE 3. WET WEIGHT OF ROOT-NODULE BACTERIA PER 100 ml FOR CULTURES RECEIVING EQUIVALENT MIXED β-RAY AND Ra OR ^{210}Po α-RAY DOSES

Days of culturing	Control	β-emitters (^{210}Po fission fragments) 5 mc/l.		Ra, 0·45 mc/l.		^{210}Po, 0·7 mc/l.	
	Weight (g)	Weight (g)	% of control	Weight (g)	% of control	Weight (g)	% of control
1	—	0·2348	—	0·2450	—	0·0678	—
2	0·3472	0·3590	103	0·3878	112	0·4536	130
5	0·6666	0·6942	104	0·6808	102	0·5716	85
9	0·9426	1·0636	113	0·8754	93	0·7054	75
16	1·1118	1·3140	119	0·1890	106	0·7314	65

TABLE 4. NUMBER OF CELLS AND TOTAL DRY CELL WEIGHT FOR *Azotobacter chroococcum* AT VARIOUS β-EMITTER AND ^{210}Po CONTENTS ON AGAR MEDIA

Emitter	mc/l.	Cells/ml		Total dry cell weight per 25 ml	
		No.	% of control	Weight (g)	% of control
Control	—	$7{\cdot}58\times10^{8}$	100	0·0284	100
Control	—	$7{\cdot}58\times10^{8}$	100	0·0284	100
Mixed β-emitters	5	$6{\cdot}99\times10^{8}$	92	0·0192	68
	25	$4{\cdot}61\times10^{8}$	61	0·0192	68
	125	$2{\cdot}56\times10^{8}$	0·034	0·0000096	0·36
^{210}Po	0·6	$3{\cdot}61\times10^{8}$	48	0·0129	45
	3	$7{\cdot}44\times10^{7}$	9·8	0·0031	10·9
	14	0	0	0	0

TABLE 5. AMOUNTS OF NITROGEN FIXED BY *Azotobacter chroococcum* RECALCULATED TO THE TOTAL VOLUME OF SUSPENSION FROM THE SURFACE AT VARIOUS β-EMITTER AND ^{210}Po CONTENTS ON AGAR MEDIA

Emitter	mc/l,	Total N		Protein N			Residual N		
		mg	% of control	mg	% of control	% of total N	mg	% of control	% of total N
Control	—	33·9	100	30·18	100	89	3·72	100	11
Mixed	5	24·4	72	22·68	75	93	1·72	46	7
β-emitters	25	22·5	66	22·15	74	99	0·35	9·4	1
	125	1·59	4·7	0	0	0	2·74	74	100
^{210}Po	0·6	16·7	49	13·32	44	79	3·38	91	21
	3	4·17	12	3·16	10	76	1·01	27	24
	14	0	0	0	0	0	0	0	0

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SUMMARY

(1) The results show that α-particles depressed the development of bacteria much more than did β-particles. This agrees with published data.

(2) When ^{210}Po was used at concentrations such as to give doses equivalent to those producing stimulation with β-rays, it greatly depressed the development of root-nodule bacteria and *Azotobacter*.

(3) A mixed α- and β-emitter (Ra), used in the medium at concentrations such as to give doses equivalent to those above, gave an intermediate effect; it can stimulate somewhat after depressing development more or less strongly.

(4) Energy production and nitrogen fixation were more sensitive to α-rays than to β-rays.

Translated by J. E. S. BRADLEY

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THE X- AND PO α-IRRADIATION DOSE CURVES FOR *B. COLI COMMUNIS**

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MANY facts have recently been discovered that cast a new light on the rôle of indirect radiation effects in radiobiology. These demand that we reconsider the theory of direct action, in particular target theory [1]. Among these facts are:

(1) the relationship between bound and free water in living organisms [2] and in aqueous protein solutions [3]. The bound water constitutes about 40 per cent of the total water, and the action of radiation on the bound water can be interpreted as a direct action.

(2) The oxygen effect found on irradiating anhydrous polymers and enzymes [4, 5] and deep-frozen micro-organisms [6].

(3) The effect of oxygen on the formation of paramagnetic centres in seeds irradiated dry [7].

(4) The effects of deep-freezing when irradiating dry materials and cells (polymer [3]; enzymes [9]; dry bacteriophage [10]; and dry seeds [11, 12].

(5) Energy migration in irradiated polymers [4] in complex compounds [13, 14] and anhydrous solutions of organic compounds (cyclohexane in benzene) [15].

Before the theory of indirect action was developed the so-called target theory was usually employed. This was based on calculating the probability of hits in a structure or system vital to cellular functioning. The structure or system is the "target" in the full physical sense of the word. In target theory we determine the number of hits required to inflict damage, which number depends on the specific ionization, and on the time factor [16, 17], and analyse the dose curve to give the number of hits required. A single-hit target gives the exponential curve, while a multi-hit target gives an S-shaped one; the slope at the inflexion becomes greater the larger the number of hits required. It has, for instance, been shown that micro-organisms usually act as single-hit targets, since exponential curves are obtained, as with *B. coli* and *S. aertryne*, and with spores of *B. mesenthericus* [16]. On the other hand the very simple mould *Aspergillus terreus* gives multi-hit effects since its X-ray dose curves are S-shaped. That this is a multi-hit effect is also shown by the fact that irradiation with Po α-particles (which have a high track density) gives an exponential curve [18, 19]. This has been interpreted as meaning that the high track density given by the α-particles produces several hits on the target simultaneously from one α-particle.†

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† The linear energy loss will in future be expressed in terms of the specific ionization, i.e. the number of ion pairs per μ of track (assuming an average of 32·5 eV per ion pair), as is commonly done in target theory [16, 17], although the formation of ions under these circumstances requires special consideration [20].

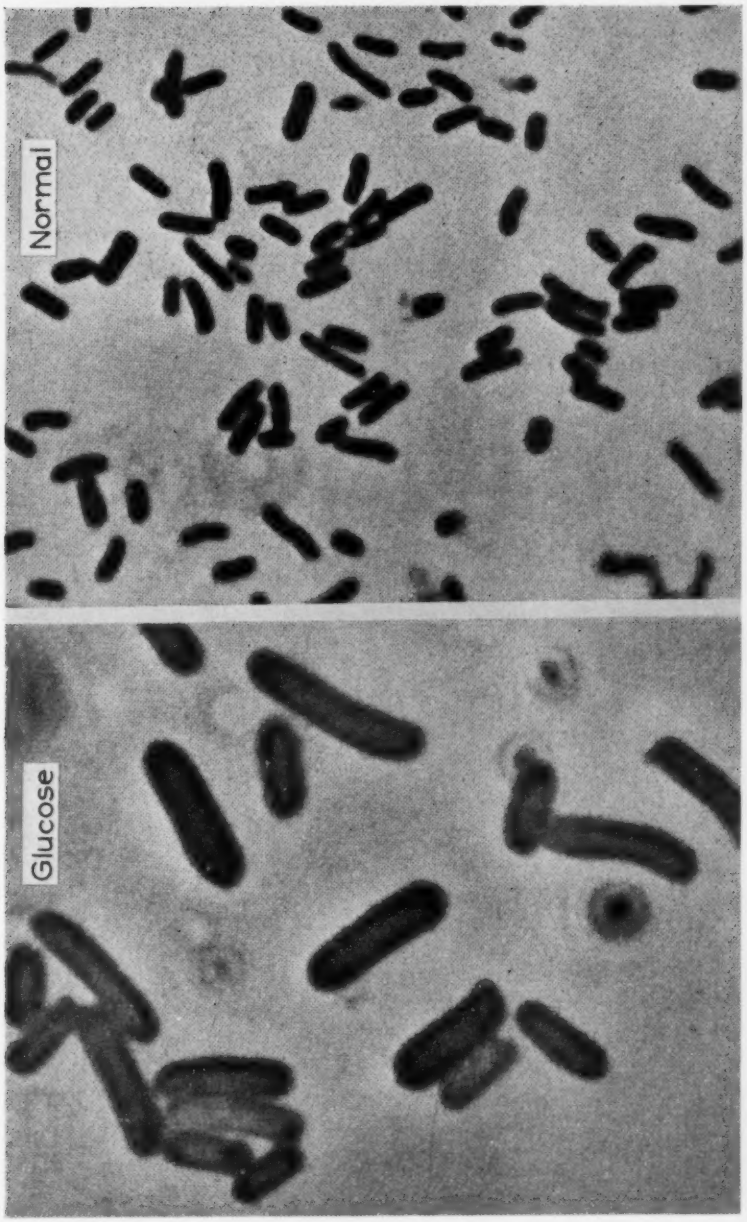

FIG. 1. Microphotographs of *B. coli* cultures ×7200. (1) "Glucose" culture. (2) "Normal" culture.

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The dose curves given by micro-organisms are not always exponential [21], as Lea would have [16], but sometimes depend on the state of the medium, the presence of oxygen, the composition of the medium, etc. *B. coli* cultures grown in anaerobic conditions or with glucose added to the medium give S-shaped curves in the absence of oxygen. Target theory would interpret changes in the dose curve as due to changes in the properties of the target. The relation between curve shape and number of hits required, as assumed in target theory, was studied on *B. coli* cultures subjected to radiations of differing specific ionization, namely X-rays with $\lambda = 0{\cdot}55$ Å and Po α-rays with $E = 5{\cdot}298$ MeV. We would expect an exponential type of curve in all cases when the α-particles are used. The 0·55 Å X-rays give about 45 ion pairs per μ along the electron tracks, whereas 5·298 MeV α-particles give 3750 pairs per μ. Hence one α-particle is sufficient to damage a cell, even if of multiple-hit type.

METHODS AND RESULTS

Normal meat-peptone cultures (1% peptone, 2% agar, pH 7·0) and the same plus 2% glucose were used with *B. coli*. Water suspensions were irradiated; after irradiation the cells were cultured on normal meat-peptone agar in petri dishes at 37°C. The colonies were counted after 24 hr. The X-irradiations were done on vials containing water suspensions with 200 bacteria per cm^3, checked photometrically from the absorption at 260 mμ. Concentration changes in the range 1: 1200 had no effect on the radio-sensitivity. Agar columns 9 mm in diameter containing 7000 bacteria per cm^3 were used with the centres of the columns 5 mm from the α-particle source.

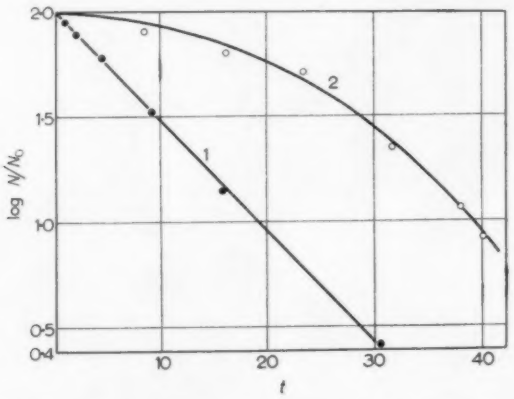

FIG. 2. Dose curves for X-irradiated *B. coli*. N/N_0 is the survival (as percentage of control). (1) Normal culture, (2) culture with glucose added.

Fig. 1 shows that the glucose cultures gave cells some 2·6 times larger in linear dimensions (18 times larger by volume) than the normal cultures. The glucose cultures were much the less radio-sensitive. The X-irradiated normal cultures gave exponentail curves with $LD_{50} = 4$ kr, and the glucose ones S-shaped curves with $LD_{50} = 16$ kr, just as in [21] (Fig. 2). The normal cultures gave exponential curves with α-particles as well, but the glucose ones gave S-shaped curves, contrary to target

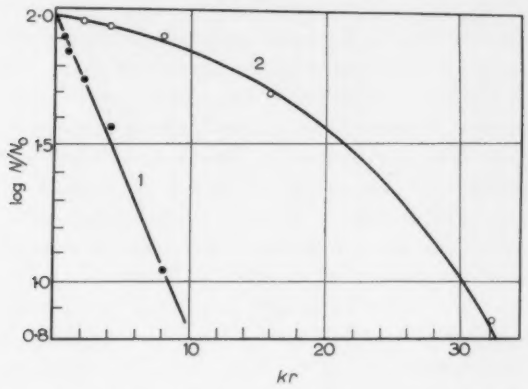

FIG. 3. Dose curves for *B. coli* irradiated with Po α-rays. N/N_0 is the survival (as percentage of control); t is the irradiation time, in min. (1) Normal culture, (2) culture with glucose added.

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theory (Fig. 3). These properties of the glucose cultures are not passed on, since on subculturing on normal medium the normal effects return. Fig. 4 shows results for a glucose culture which was retransferred to a normal one; the cultures in this case were irradiated with ^{60}Co γ-rays at dose rates of about 600 r/min. The initial and final normal cultures were of just the same radio-sensitivity, while the glucose culture was much less sensitive. The first two cultures gave exponential curves with X-rays, while the glucose gave an S-shaped one. The number of hits required (calculated by Jordan's method [17]) comes out as $n=3$ for X-rays.

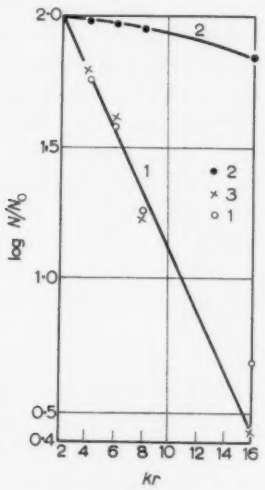

FIG. 4. Dose curves for X-irradiated *B. coli*. N/N_0 is the survival (as percentage of control). (1) Initial normal culture, (2) the same culture after subculturing on the glucose medium, (3) the same culture after subculturing again on the normal medium.

DISCUSSION

Polonium α-particles have a specific ionization some eighty times larger than 0·55 Å X-rays. The energy dissipated by the passage of one α-particle through a cell should be sufficient no matter how many hits are required; an exponential curve is therefore to be expected. However, the glucose culture gives an S-shaped curve with $n=3$ with α-particles. The curve shape is therefore not correlated with the number of hits, which disagrees with classical target theory. The shape of the curve must be closely related to the way radiation interacts with matter, in relation both to the direct and to the indirect effects; analysis of the interaction mechanism is of fundamental importance to the theory of primary radiobiological effects. The classical target theory clearly fails to make proper allowance for certain biological factors which influence the shape of the dose curve. These factors include the differing rôles of the nuclear nucleoproteins and of the cytoplasmic proteins, since the first play a decisive part in cell division, while the second are mainly involved in enzyme processes and metabolism. The nucleoproteins are much more radio-sensitive than the others; they show chain breakage and depolymerization on irradiation. The nucleoprotein structures are unique, so that even local damage to a single structure can profoundly disturb protein synthesis, resulting ultimately in failure to divide, and death, particularly at the division stages.

The effects of ionizing radiations (α-particles, protons, electrons) on single-celled organisms should follow stochastic laws, because the damage is localized and the nucleoprotein structures unique; the dose curve then relates to a Poisson distribution, and target theory should be applicable. The cytoplasmic proteins do not have unique structures though, and damage to some protein molecules need not disrupt the activity of the cell, if the damage can be accommodated by redistribution of the enzyme functions. Cell death due to cytoplasmic damage only occurs if the damage exceeds some limit of tolerance, so here the shape of the dose curve is controlled by the degree of the irreversible damage, and a threshold should be apparent; this threshold should lie at high doses as the cytoplasmic proteins are of low sensitivity.

Thus nucleoprotein structures (1) have high radio-sensitivity, (2) when damaged, prevent the cell dividing while metabolism continues, (3) show radiation damage of stochastic type, so the dose curve shape and number of hits required are directly related, as in classical target theory. On the other hand, cytoplasmic proteins (1) have low radio-sensitivity, (2) only give cell death if extensively damaged, (3) show no effect if the damage tolerance is not exceeded. This hypothesis is confirmed by the following facts.

(1) The power to divide is much more easily lost than the power to metabolize in yeasts and bacteria.

(2) Giant forms appear when radiations act at low dose rates for long times, so the power to divide is lost while growth and metabolism continue.

(3) Polyploid cells and organisms are much less radio-sensitive than haploid ones [22].

(4) The dose response curve for multiplication is exponential [16], whereas that for loss of enzyme activity is S-shaped, with a marked threshold [23].

We used the power to divide; although nuclei have not been demonstrated as separate structures in *B. coli* or in most other micro-organisms, submicroscopic

nucleoprotein structures of unique nature directly related to cell division may be present. The dose curves obtained with the normal and glucose cultures can then be interpreted as arising from the superposition of nucleoprotein and cytoplasmic effects. Normal *B. coli* cultures show the nucleoprotein effect, and the capacity to divide is lost before cytoplasmic damage becomes apparent. The radio-sensitivity is therefore high and the dose curve agrees with target theory. The nucleoprotein effect plays less part in the glucose cultures, perhaps because the larger cells contain more nucleoprotein structures. It is as if the unique nucleoprotein structures were duplicated as in polyploidy, so that local damage to one would not remove the power to divide.* The radio-sensitivity, as estimated from the survival, is therefore reduced, and the effects of cytoplasmic damage begin to predominate. The curve here is composed of the nucleoprotein and cytoplasmic ones superimposed; since the latter shows a threshold, the overall dose curve is S-shaped, no matter what the specific ionization, i.e. for both X- and α-rays. This idea could be tested by determining the contents and compositions of the nucleoprotein structures, and by studying the effects of radiation on the nucleoprotein structures and enzyme systems.

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SUMMARY

(1) *B. coli* cultures from meat-peptone agar containing 2% glucose showed cells 2·6 times as long (18 times as large) as normal.

(2) The survival curves with X-rays and Po α-rays were S-shaped for the glucose cultures, which agreed with the classical target theory.

(3) The properties of the glucose culture were not inherited, and the culture returned to normal after subculturing once on normal meat-peptone agar.

(4) The dose curves are interpreted in terms of a theory of the differing rôles of nucleoproteins and cytoplasmic proteins. The curve found with the glucose culture is interpreted as arising from the superposition of nuclear and cytoplasmic effects.

Translated by J. E. S. BRADLEY

* Zelle and Ogg's paper [24] appeared after this one had been accepted for publication. These workers exposed *Esch. coli* cells (82/r line) to camphor vapour, and produced a culture with cells three times the normal size; this culture gave an S-shaped dose curve with X-rays instead of an exponential one. The curve was analysed by Tobias's [25] method to show that a diploid culture had formed; this was confirmed by some genetic experiments involving crossing various lines.

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THE EFFECTS OF γ-RAYS ON THE ABSORPTION SPECTRA OF PYRIMIDINE AND PURINE BASES, AND OF NUCLEIC ACIDS*

T. N. RYSINA and R. E. LIBINZON

(*Received* 8 *April* 1957)

MUCH attention has been given to the effects of ionizing radiations on the nucleic acids and their parent compounds because these acids play such an important part in fundamental biological processes. The nucleic acids have been shown to be reduced in structural viscosity in aqueous solution [1–3]. Scholes and Weiss [4–6] have described the changes produced in the nucleic acids or fragments thereof by X-ray doses of 2×10^4–10^6 r. Polynucleotides were broken down and inorganic phosphate and small amounts of free purine bases were formed. Barron *et al.* [7] have found that this destruction is accompanied by a fall in the ultra-violet absorption, which was proportional to the radiation dose. The ribose and phosphate components were observed to protect the base.

These changes in ultra-violet absorption are of interest in that the amounts of compound destroyed or altered can be determined quantitatively without resort to laborious or complex analyses. The method is of particular value at very low concentrations, where normal chemical methods are very difficult to apply. We studied the dose-dependence of the spectral changes in the more important purine and pyrimidine bases, and in the nucleic acids. The way the changes were affected by the concentration was also studied.

METHODS

We used cytosine, uracil and thymine, and adenine, guanine, xanthine and uric acid at from 5×10^{-5} M to 10^{-2} M; ATP at 5×10^{-5} to 10^{-3} M; and RNA and DNA (sodium salts) at 0·003–0·03 per cent. Volumes of 5 ml were irradiated with ^{60}Co

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γ-rays in glass test-tubes, at doses of 1–200 kr and dose rates of 168–176 r/min. The doses were determined by ferrous sulphate dosimetry [8, 9]. In no series did the doses to the individual tubes vary by more than ±10 per cent.

The spectra were recorded on an SF-4 spectrophotometer. The solutions were diluted by factors of 5, 10 or 100, in accordance with the initial concentrations, to

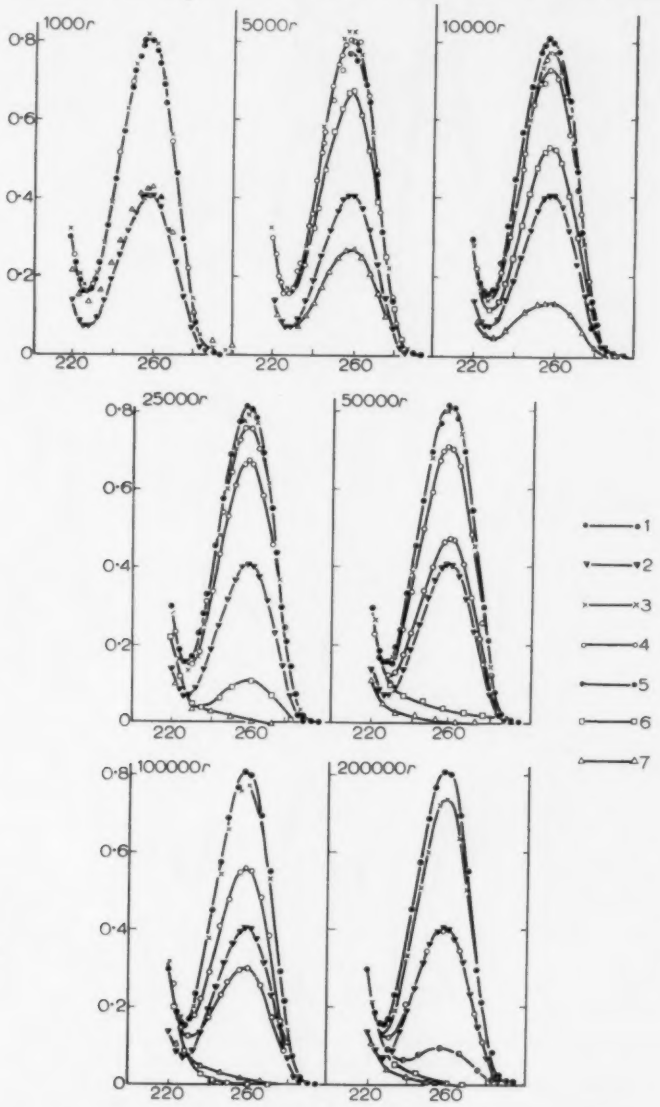

FIG. 1. Effects of γ-ray doses of 10^3–2×10^5 r on the absorption spectra of uracil solutions of various concentrations. Abscissae: wavelenth in mμ. Ordinate: optical density. Control solutions 10^{-4} M (1) and 5×10^{-5} M (2). Irradiated solutions 10^{-2} M (3), 10^{-3} M (4), 5×10^{-4} M (5), 10^{-4} M (6) and 10^{-5} M (7).

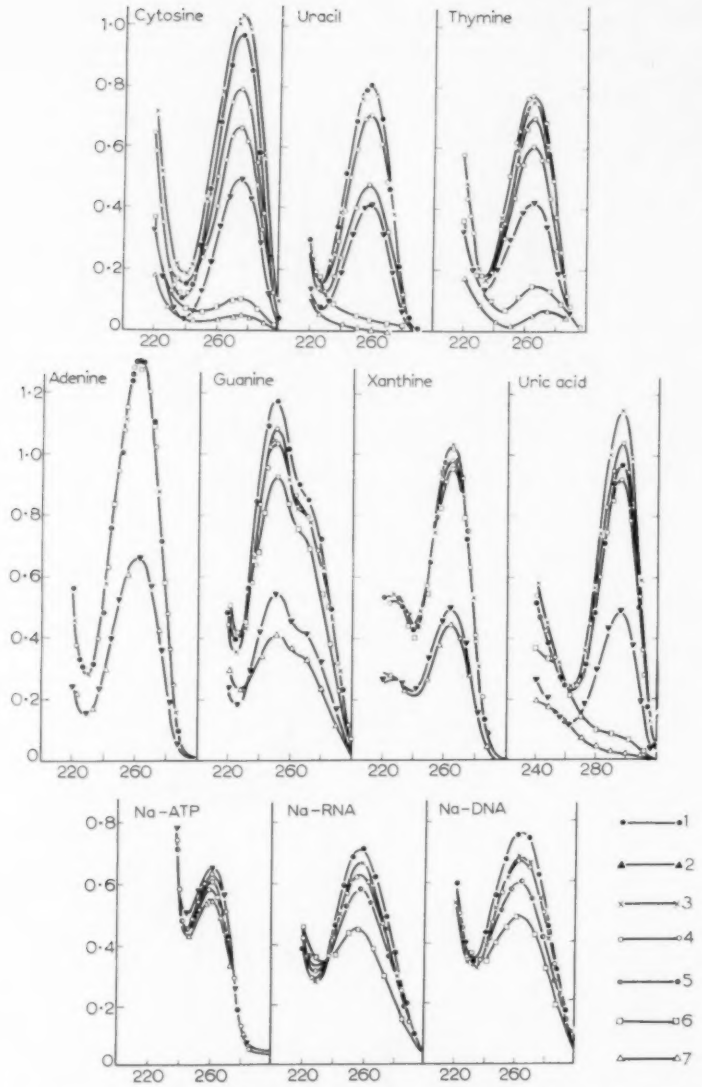

FIG. 2. Effects of irradiation on the absorption spectra of solutions of purine and pyrimidine bases, and of nucleic acids. Dose: 50,000 r of γ-rays. Abscissa: wavelength in mμ. Ordinate: optical density. The symbols for the absorption spectra of solutions of purine and pyrimidine bases, and of Na-ATP are as in Fig. 1. The control solutions for Na-RNA and Na-DNA were 0·003% (1), and the irradiated ones 0·03% (3), 0·015% (4), 0·006% (5) and 0·003% (6).

give a final equivalent concentration of 10^{-4} M. The optical density was read at 2 mμ intervals over the 220–300 mμ range using 1 cm cells. The curves for fresh and irradiated solutions were compared.

RESULTS

The ultra-violet absorptions of the bases were reduced by irradiation, particularly at the absorption maxima. Fig. 1 shows curves for uracil at various concentrations and doses; the fall in optical density clearly increases with the dose. The effect clearly also depends on the concentration. At 1000 r the maximum is hardly affected at the lowest concentration (5×10^{-5} M). At 5000 r the effect can be seen at 10^{-4} and 5×10^{-5} M, and at 10,000 r in all cases. The optical density approaches zero at 50–200 kr in the 5×10^{-5} and 10^{-4} M solutions.

The same effect is found in all other cases; much of the data has had to be omitted, of course, and Fig. 2 shows only specimen curves for all the compounds used, at 50,000 r. The relative radiosensitivities can be judged roughly from these curves. The precise effects are determined by comparing the spectra of fresh and irradiated solutions; the optical densities of the latter are expressed as percentages of the initial values in Fig. 3. The compounds clearly differ in radio-sensitivity. The pyrimidines showed greater falls at the maxima than did the purines, except for uric acid, which is highly sensitive. The sodium salt of ATP is more resistant than adenine, while RNA and DNA, as their sodium salts, do not show any essential differences in behaviour.

The dependence of the effect on the concentration is marked, the lower concentrations showing the larger effect. The optical density approaches zero at 50–200 kr in the 5×10^{-5} and 10^{-4} M solutions for cytosine, uracil, thymine and uric acid.

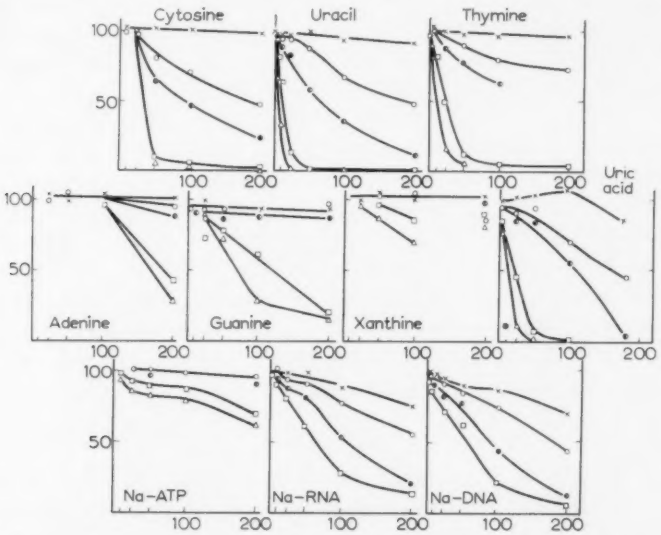

FIG. 3. Reductions in the optical densities of solutions of purine and pyrimidine bases, and of nucleic acids on irradiation. Abscissa: γ-ray dose, kr. Ordinate: optical density as per cent of initial value. Notation as in Fig. 2.

DISCUSSION

The data of Cavalieri *et al.* [10, 11] indicate that the purine and pyrimidine bases have the following chromophores:

$$\begin{array}{c} | \quad | \quad | \\ -C-C-C=N- \end{array} \quad \text{and} \quad \begin{array}{c} | \quad | \quad | \\ -C=C-C=O \end{array}$$

(both in the pyrimidine rings). These are considered as the main chromophores, but the spectra are really determined by their interactions with all the rest of the molecule. The falls in ultra-violet absorption are mainly caused by disruption of the chromophores (rupture of the pyrimidine rings is one possibility). Other changes in the molecules, e.g. deamination, or loss of double bonds, or imidazole ring rupture, will have similar but lesser effects.

Scholes and Weiss's data [4–6] indicate that these changes occur in nucleic acids, nucleotides, nucleosides, pyrimidines and purines. The lesser radio-sensitivity of the purines is explained by the protective action of the imidazole ring. Similar effects produced by additional groups in the molecule are found in other cases; thus thymine (5-methyl uracil) is somewhat more resistant than uracil, perhaps because of its CH_3 group. The ribose and phosphate may also protect ATP.

Redox processes cause chemical changes in irradiated solutions, including changes responsible for the effects noted here. Enzymatic oxidation of adenine and guanine gives uric acid, the primary products being hypoxanthine and xanthine respectively. One naturally assumes the same changes to occur on irradiation; simple calculations on the spectra show that xanthine is formed from guanine on irradiation. Using 10^{-4} M solutions, 5000 r gives $0{\cdot}54 \times 10^{-2}$ μM xanthine per ml, while 50,000 r gives $0{\cdot}95 \times 10^{-2}$ μM/ml and 200,000 r $1{\cdot}63 \times 10^{-2}$ μM/ml. That the compound was xanthine was confirmed chromatographically. The changes in optical density with guanine are therefore composed of the difference between guanine consumed and xanthine formed. No appreciable amount of uric acid was found in the irradiated guanine solutions, probably because uric acid is easily oxidized.

The rapid drop in the optical density on irradiating uric acid solutions may occur because the radiolysis products from water oxidize the uric acid to allantoin or alloxan, both of which contain no chromophores and therefore give no specific absorption in the ultra-violet. This mechanism was demonstrated by irradiating (45 kr) frozen uracil solutions of various concentrations; no effect occurred. The effects on the liquid solutions at this dose were considerable (Fig. 3).

The pyrimidines and purines are almost completely destroyed in dilute (5×10^{-5} to 10^{-4} M) solutions at high γ-ray doses (50–200 kr). This is biologically very significant, since the free purine and pyrimidine concentrations in animal tissues are low and, at a few μg/g of tissue, do not exceed 10^{-4} M [12].

SUMMARY

Doses of from 1000 to 200,000 r of ^{60}Co γ-rays reduce the ultra-violet absorption of the biologically most important purines and pyrimidines, and also of the nucleic acids, partly at the lower concentrations (5×10^{-5} to 10^{-4} M). The loss in optical density increases with the γ-ray dose. Additional groups attached to the purine or pyrimidine rings decrease the radio-sensitivities. *Translated by* J. E. S. Bradley

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METHODS AND APPARATUS

THE DOSIMETRY OF IONIZING RADIATIONS OF FINITE RANGE*

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1. THE BASIS OF THE NEW METHOD OF CALCULATING THE INTEGRAL ABSORBED DOSE

PURE β-emitters have formed a large fraction of the artificial radioisotopes that have been used. Methods are required for dosimetry calculations for such emitters, which have particles of finite range. The solutions available for γ-rays cannot be applied to β-rays, and no general solution is at present available for the latter. Kononenko [1] has solved several problems occurring in the dosimetry of α-particles. The method presented below is a new one, in which the finite range of the particles is allowed for. In essence it amounts to replacing the normal dose calculation by the calculation of the total range of the particles within the object. Its particular advantage is that the finiteness of the range is taken into account in a comparatively simple and direct fashion. The idea on which it is based arose from some calculations in relation to Glocker's [2] criticism,† when it appeared that in one particular case the total range of the particles within the object agreed, apart from a constant factor, with Souttar's [3] formula‡ for the dose at a point due to a sphere uniformly filled with radon (see below).

The method is applicable to any body irradiated by any source, but, as is often

* Biofizika **3:** No. 4, 494–515, 1958.

† The reasons for posing the problem are also given by Glocker.

‡ Dose $= k\,(r\rho+\frac{1}{2}\,(\rho^2-r^2)\ln[(\rho+r)/(\rho-r)]$, where r is the radius of the sphere and ρ is the distance at which the electron is ejected from the centre of the sphere.

done in dosimetry calculations, we restrict ourselves to bodies which are figures of rotation irradiated by point sources lying on the rotation axes.

The main assumptions are briefly as follows:

(1) The source is assumed to emit uniformly in all directions in all respects. In future we shall replace the assembly of ranges actually occurring by the mean range, which is, in view of the above assumption, the same in all directions and denoted by R=const. This range R is only made constant for the purposes of the calculation; the real continuum of a β-emitter is not replaced by a monoenergetic emission.

The *total internal range* is the integral of those parts of the mean range which lie within the object. This internal range depends on the shape of the body and on the direction considered (i.e. on the angle formed with the symmetry axis); the mean range when the particle passes right through the object and when it stops in it can be considered as a statistical characteristic of the radiation spectrum.

(2) The reduction in the energy flux resulting from the fall-off in the number of particles and from the reduction in particle energy is calculated as a function of β-particle energy and of the stopping power of the medium from experimental data. The experimental attenuation factor represents an average over the values belonging to the various components of the β-spectrum. We assume (*a*) that the attenuation can be assumed to be exponential to an accuracy sufficient for practical purposes, subject to the proviso that the β-particle range is finite, (*b*) that the media around and within the object have different stopping powers, (*c*) that the experimental attenuation coefficient μ is the same in all parts of the object, and changes at the boundary with the external medium.

(3) The energy reaching any point in the object is calculated with due allowance for the losses occurring both before and after the particle reaches the object, and with due allowance for the way the tracks are distributed within the object. The results are integrated over all directions lying within the solid angle which the object subtends at the source. The β-particles are considered as travelling in straight lines, since the attenuation coefficients are defined on this basis.

We must point out here one assumption which reduces the accuracy of the calculations, but which we are forced to make in order to avoid excessively complex mathematical operations. Since the mean range R depends on the stopping powers of the media [4–7], the value must be affected by the way the range is distributed between the various media; hence R must vary somewhat with the direction considered. We have neglected these variations and taken R as constant and independent of direction. The error this occasions is small if (*a*) the stopping powers of the internal and external media differ little (if $\mu-\mu_1$ is small), (*b*) the solid angle subtended at the source by the part of the body in which the particles come to rest is small, (*c*) the ratio of the internal and external paths is large or small.

The principle of interchangeability of source and object [8] indicates that the solutions derived below for a point source are also solutions for applications in the form of bodies of rotation for points lying on the rotation axes. The treatment is readily extended to bodies irradiated by extended sources if these lie entirely on the symmetry axes of the object, i.e. if the object is a sphere or the source a rod lying on a symmetry axis.

The method is broken down into its separate stages, to make the process clear; first of all we consider the spatial distribution of the emission from a point source, and then introduce a figure of rotation with its axis passing through the source.

Consider a thin spherical shell of thickness dy and radius y surrounding the source, assuming no absorption in the intervening space. If the source is of activity M (in mc) the energy absorbed by the shell per hour is

$$\begin{aligned}&(M\times 3{\cdot}7\times 10^{7}\bar{E}_{\beta}\times 3600\times 1{\cdot}602\times 10^{-6}(1-\exp^{(-\mu)})dy)/100\\&\quad=2133{\cdot}864M\bar{E}_{\beta}(1-\exp^{(-\mu)})dy\\&\quad=A\,dy,\ \text{rad}\times\text{cm}^3/\text{hr}\end{aligned}$$

where $\bar{E}_{\beta}$ is the mean β-ray energy in MeV and μ the linear absorption coefficient.

When an object is introduced we have to calculate the path length in it; the calculation of total path length is complicated by the need to allow for the attenuation. All rays emitted by the source at the same angle φ to the rotation axis will have the same internal and external paths. The probability of a ray falling within the conical shell lying between φ and $\varphi+d\varphi$ (Fig. 1) is $\frac{1}{2}\sin\varphi d\varphi$. To determine the total

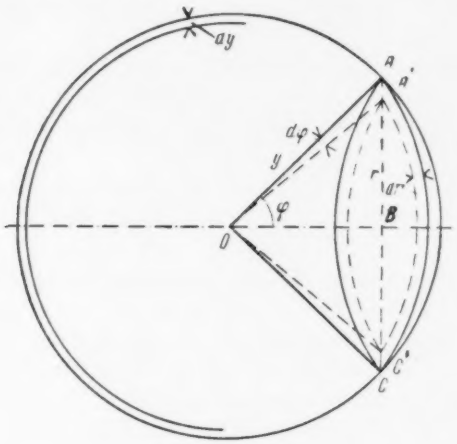

FIG. 1

internal range we need to know the external range as well. These ranges depend on the shape of the object and its distance from the source, and therefore have to be determined for each problem separately.

Let y_1 denote the distance from the source to the object along a ray, and the distance to any point within the object by y. The energy absorbed in a length dy will then be

$$2134\,M\bar{E}_{\beta}\mu\frac{\sin\varphi}{2}e^{-\mu_1 y_1}e^{-\mu(y-y_1)}dyd\varphi = \qquad \text{A}$$

$$=A\frac{\sin\varphi}{2}e^{-\mu_1 y_1}e^{-\mu(y-y_1)}dyd\varphi\quad\text{rad}\times\text{cm}^3/\text{hr}$$

Integrating with respect to y from y_1 to y_2, we get

$$2134\,M\bar{E}_{\beta}\frac{\mu}{2}\int_{y_1}^{y_2}e^{-\mu_1 y_1}e^{-\mu(y-y_1)}\sin\varphi d\varphi dy. \qquad \text{B}$$

The second integration for φ, to cover the entire region for which the dose is required, gives

$$2134\, M\bar{E}_\beta \frac{\mu}{2} \int_{\varphi_1}^{\varphi_2} \sin\varphi \int_{y_1}^{y_2} e^{-\mu_1 y_1} \cdot e^{-\mu(y-y_1)} dy d\varphi. \qquad \text{C}$$

The functions under the integral signs are independent of the detailed nature of the problem, the sole variable quantities being the limits to φ and y. The inverse square law does not appear in explicit form in the calculation, but is actually allowed for by the fall-off in the density of the points with distance. In all cases the first integration (for y) is very simple, whereas the second (for φ) can often be found only approximately; various approximate devices are used, depending on the value of μ. In the simpler cases the result is the integral exponential function, which is tabulated [9]; in other cases the exponential function is expanded in powers of μ and μ_1 (if μ and $\mu_1 < 1$). The series can be terminated at the second-order terms without loss of accuracy; if μ and $\mu_1 > 1$, integration by parts is used, as the μ's appear in the denominator (i.e. to negative powers) and the series converges rapidly. In the latter case the terms become rapidly more complex, and the series cannot conveniently be extended beyond the terms in μ^{-2}*.

All the basic problems can be solved using two of these methods, which can be applied to penetrating radiations as well as to β-rays (for which $\mu \gg 1$ usually). The problems can be divided into ones concerning plane-parallel layers, cylinders and spheres.

The object is divided up into zones for the integral dose calculation; by "zone" we mean a region of the object for which the internal path lengths are described by the same formula. The zones differ according to whether the particle stops within the layer or escapes from an edge. In the case of a cylinder the difference lies between termination at a side face or at the base. The boundaries between the zones define the integration limits for φ. The shapes and sizes of the zones depend on the distance of the object from the source, on the object itself and on the ratio of size of object to range of particle. The number of cases to be considered thus depends on the geometrical relationships (with a sphere, for instance, there are six distinct cases to be considered). The most difficult case will always be treated first in what follows, as the other cases can then be dealt with by analogy.

2. IRRADIATION OF A PLANE-PARALLEL LAYER

This problem has not so far been treated in the Soviet literature [10–15]. The layer is "thin" if some particles pass right through, and "thick" if all come to rest in the layer. The problem falls into two sections, according to whether the source lies inside or outside the layer.

Problem 1. The source lies a distance f above a thin layer of thickness h. The range is R, with $R > h+f$. The attenuation coefficients are μ (in the layer) and μ_1 (above). There are two zones; in zone 1 the particles escape from the bottom surface, and in zone 2 they come to rest in the layer.

Zone 1 (*QSP*, Fig. 2). The integration limits are determined before deriving the formula for the integral dose *Di*. The upper limit in φ is determined from triangle

* We are indebted to Professor Smirnov for pointing out how to avoid the difficulties arising for $\mu \gg 1$.

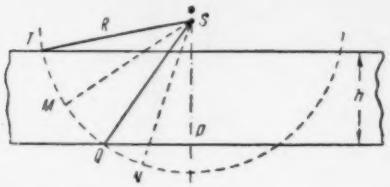

FIG. 2

SPQ as $\varphi_2=\cos^{-1}[(f+h)/R]$, the lower as $\varphi_1=0$. The limits for the internal section *SN* in the first zone are

$$y_1=\frac{f}{\cos\varphi}\ \text{and}\quad y_2=\frac{f+h}{\cos\varphi}. \qquad \text{D}$$

The integral dose formula (where $A=2134\,E_\beta\mu\,M$, for brevity) is

$$Di^S=A\int_0^{\cos^{-1}(f+h)/R}\frac{\sin\varphi}{2}\int_{f/\cos\varphi}^{(f+h)/\cos\varphi}e^{-\mu(y-f/\cos\varphi)}\;e^{-\mu_1 f/\cos\varphi}\;dyd\varphi. \qquad \text{E}$$

Changing the variable, we have

$$\frac{f}{\cos\varphi}=x;\ \cos\varphi=\frac{f}{x};\ \sin\varphi d\varphi=\frac{fdx}{x^2}. \qquad \text{F}$$

The limits for x are $x=f$ when $\varphi=0$ and $x=fR/(f+h)$ when $\varphi=\cos^{-1}[(f+h)/R]$ (first integral), and x (lower) and $x(f+h)/f$ (upper).

$$Di_1^S=A\frac{f}{2}\int_f^{Rf/(f+h)}\frac{1}{x^2}\cdot\int_x^{x(f+h)/f}e^{-\mu(y-x)}\;e^{-\mu_1 x}\;dydx$$

$$=A\frac{f}{2\mu}\int_f^{fR/(f+h)}\frac{1}{x^2}\;(e^{-\mu x}-e^{-\mu x(f+h)/f})\;e^{\mu x}\;e^{-\mu_1 x}\;dx$$

$$=A\frac{f}{2\mu}\int_f^{fR/(f+h)}\frac{1}{x^2}\;(1-e^{-\mu xh/f})\;e^{-\mu_1 x}\;dx. \qquad \text{(a)}$$

The integral can be found approximately either by expanding the integrand in series form (if $\mu<1$), or can be referred to the integral exponential function, which is tabulated. We commence with expanding the integrand. We expand $\exp(-\mu xh/f)$ and $\exp(-\mu_1 x)$ separately, extract μ from under the integral sign, and stop the series at terms in μ^2, μ_1^2 and $\mu\mu_1$, and get
for zone 1

$$Di_1^S=A\frac{f}{2}\left\{\frac{h}{f}\ln\frac{R}{f+h}-\mu\frac{h^2}{2f}\left(\frac{R}{f+h}-1\right)-\mu_1 h\left(\frac{R}{f+h}-1\right)\right.$$
$$\left.+\mu^2\frac{h^3}{12f}\left(\frac{R^2}{(f+h)^2}-1\right)+\mu_1^2\frac{fh}{4}\left(\frac{R^2}{(f+h)^2}-1\right)+\mu\mu_1\frac{h^2}{4}\left(\frac{R^2}{(f+h)^2}-1\right)\right\}, \qquad \text{G}$$

for zone 2

$$Di_{II}^{S} = A \int_{\cos^{-1}(f+h)/R}^{\cos^{-1} f/R} \frac{\sin\varphi}{2} \int_{f/\cos\varphi}^{R} e^{-\mu\left(y-\frac{f}{\cos\varphi}\right)} e^{-\mu_1 f/\cos\varphi}\, dy d\varphi =$$

$$= A\frac{f}{2}\left\{\frac{h}{f} + ln\frac{f+h}{f} - \mu\left[\frac{Rh}{2f} + \frac{Rh}{2(f+h)} - Rln\frac{f+h}{f}\right]\right. \qquad \text{H}$$

$$- \mu_1\left[-\frac{Rh}{f+h} + Rln\frac{f+h}{f}\right] + \mu^2\left[\frac{R^2h}{6f} + \frac{R^2h}{2(f+h)} - \frac{R^2h(2f+h)}{12(f+h)^2}\right]$$

$$\left. + \mu_1^2\left[\frac{R^2h}{2(f+h)} - \frac{R^2h(2f+h)}{4(f+h)^2}\right] + \mu\mu_1\left[-\frac{R^2h}{f+h} + \frac{R^2h(2f+h)}{4(f+h)^2} + \frac{R^2}{2}ln\frac{f+h}{f}\right]\right\}.$$

The integral dose is found by summing for the two zones

$$Di_{I.1}^{S} = A\frac{f}{2}\left\{\frac{h}{f} + \frac{h}{f}ln\frac{R}{f} + \frac{f-h}{f}ln\frac{f+h}{f} - \mu\left[\frac{(2R-h)h}{2f} - Rln\frac{f+h}{f}\right] -\right.$$

$$- \mu_1\left[-h + Rln\frac{f+h}{f}\right] + \mu^2\left[\frac{R^2h(h-f)(h-2f)}{4f(f+h)^2} - \frac{h^3}{12f} - \frac{R^2}{2}ln\frac{f+h}{f}\right] +$$

$$\left. + \mu_1^2\left[\frac{R^2h}{4(f+h)} - \frac{fh}{4}\right] + \mu\mu_1\left[-\frac{R^2h}{2(f+h)} - \frac{h^2}{4} + \frac{R^2}{2}ln\frac{f+h}{f}\right]\right\}. \qquad (1)$$

The solutions for particular cases are easily derived from this general formula. If we put $f=0$ (contact irradiation), then, extricating f and transforming the logarithms, we have

$$Di_{I.1}^{S} = A\frac{h}{2}\left\{1 + ln\frac{R}{h} - \mu\frac{2R-h}{2} + \mu^2\frac{3R^2-h^2}{12}\right\}. \qquad (1a)$$

All terms containing μ_1 drop out.

If we put $\mu=\mu_1=0$, i.e. neglect the energy losses, then

$$Di_{I.1}^{S} = A\frac{h}{2}\left\{1 + ln\frac{R}{h} - \frac{f+h}{f}ln\frac{f+h}{f}\right\}. \qquad (1b)$$

If we put $\mu=\mu_1\neq 0$, i.e. if we have an intermeditae medium of the same stopping power, (1) becomes much simpler.

$$Di_{I.1}^{S} = A\frac{f}{2}\left\{\frac{h}{f} + \frac{h}{f}ln\frac{R}{f} + \frac{f+h}{f}ln\frac{f+h}{f} - \mu\left[\frac{(2R-h)h}{2f} - h\right]\right.$$

$$\left. + \mu^2\left[\frac{(3R^2-h^2)h}{12f} - \frac{h}{4}(f+h)\right]\right\}. \qquad (1c)$$

Using the second method of approximation, i.e. putting the integral in (a) in the integral exponential form, we have for zone 1

$$Di_{1}^{S} = A\frac{f}{2\mu}\left\{\mu_1\Big|_{\mu_1 Rf/(f+h)}^{\mu_1 f}\left[\frac{e^{-z}}{z} + Ei(-z)\right]\right. \qquad \text{I}$$

$$\left. + \frac{\mu h + \mu_1 f}{f}\Big|_{R(\mu h+\mu_1 f)/(f+h)}^{\mu h+\mu_1 f}\left[\frac{e^{-z}}{z} + Ei(-z)\right]\right\}.$$

For zone 2

$$Di_{II}^{S} = A\frac{f}{2\mu}\left\{\mu_1 \left|_{\mu_1 R}^{\mu_1 Rf/(f+h)} \left[\frac{e^{-z}}{z} + Ei(-z)\right]\right.\right. \cdot$$
$$\left.\left. -(\mu_1-\mu)\,e^{-\mu R}\left|_{(\mu_1-\mu)R}^{(\mu_1-\mu)Rf/(f+h)} \left[\frac{e^{-z}}{z} + Ei(-z)\right]\right.\right\}. \qquad \text{J}$$

The complete solution for a thin layer is then

$$Di_{I.1}^{S} = A\frac{f}{2\mu}\left\{\mu_1 \left|_{\mu_1 Rf/(f+h)}^{\mu_1 t} \left[\frac{e^{-z}}{z} + Ei(-z)\right] + \frac{\mu h+\mu_1 f}{f}\right.\right. \left|_{\mu h+\mu_1 f}^{(\mu h+\mu_1 f)R/f} \left[\frac{e^{-z}}{z}\right.\right.$$
$$\left.\left. + Ei(-z)\right] + \mu_1 \left|_{\mu_1 R}^{\mu_1 Rf/(f+h)} \left[\frac{e^{-z}}{z} + Ei(-z)\right]\right.\right.$$
$$\left.\left. + (\mu_1-\mu)\,e^{-\mu R}\left|_{(\mu_1-\mu)Rf/(f+h)}^{(\mu_1-\mu)R} \left[\frac{e^{-z}}{z} + Ei(-z)\right]\right.\right\}. \qquad \text{K}$$

Problem 2. The source S lies at a height f above a thick layer (Fig. 3). This problem is much simpler, because there is only one zone. The layer thickness does not enter into the calculation.

$$Di_{I.2}^{S} = A\int_0^{\cos^{-1} f/R} \frac{\sin\varphi}{2} \int_{f/\cos\varphi}^{R} e^{-\mu(y-f/\cos\varphi)}\; e^{-\mu_1 f/\cos\varphi}\, dy d\varphi$$
$$= A\frac{f\mu}{2}\int_f^R \frac{1}{x^2}\int_x^R e^{-\mu(y-x)} e^{-\mu_1 x} dy dx. \qquad \text{L}$$

Substitution and simple transformations give

$$Di_{I.2}^{S} = A\frac{f}{2}\left\{\frac{R-f}{f} - ln\frac{R}{f} - \mu\left[\frac{R^2-f^2}{2f} - Rln\frac{R}{f}\right] - \mu_1\left[-(R-f)+Rln\frac{R}{f}\right]\right.$$
$$+ \mu^2\left[\frac{(R-f)^2}{4} + \frac{R^3-f^3}{6f} - \frac{R^2}{2}ln\frac{R}{f}\right] + \mu_1^2\frac{(R-f)^2}{4}$$
$$\left. + \mu\mu_1\left[-\frac{(R-f)(3R-f)}{4} + \frac{R^2}{2}ln\frac{R}{f}\right\}\right]. \qquad (2)$$

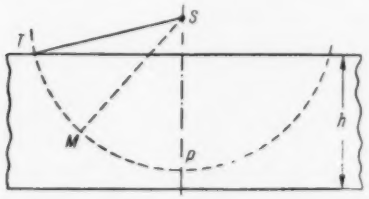

FIG. 3

If $f=0$ (contact irradiation) we have

$$Di_{I.2}^{S} = A\frac{1}{2}\left\{R - \mu\frac{R^2}{2} + \mu^2\frac{R^3}{6}\right\}, \text{ or more precisely}$$

$$Di_{I.2}^{S} = A\frac{1}{2\mu}\left(1 - e^{-\mu R}\right) = A\frac{1}{2}\int_0^R e^{-\mu x}\, dx. \qquad (2a)$$

If $\mu=\mu_1=0$ then

$$Di^S_{l.2} = A\frac{1}{2}\left(R - f - f\ln\frac{R}{f}\right). \tag{2b}$$

If $\mu=\mu_1\neq 0$ then

$$Di^S_{l.2} = A\frac{f}{2}\left\{\frac{R-f}{f} - \ln\frac{R}{f} - \mu\frac{(R-f)^2}{2f} + \mu^2\frac{(R-f)^2(2R+f)}{12f}\right\}. \tag{2c}$$

The integral in (b) is easily referred to the integral exponential function

$$Di^S_{l.2} = A\frac{f}{2\mu}\left\{\int_f^R \frac{1}{x^2}e^{-\mu_1 x}\cdot dx - e^{-\mu R}\int_f^R \frac{1}{x^2}e^{-(\mu_1-\mu)x}\cdot dx\right\} \qquad \text{M}$$

$$= A\frac{f}{2\mu}\left\{\mu_1\left|_{\mu_1 R}^{\mu_1 f}\left[\frac{e^{-z}}{z} + Ei(-z)\right] + (\mu_1-\mu)e^{-\mu R}\right|_{\mu_1 R}^{\mu_1 f}\left[\frac{e^{-z}}{z} + Ei(-z)\right]\right\}.$$

When the source lies within the medium no account is needed of external absorption. The problems divide into two types, both of which can be dealt with by the solutions above for contact irradiation. The source S lies within the layer; through S we draw a plane parallel to the surfaces. The total dose received by the layer is then the sum of the doses for the two contact irradiation layers produced, which can be found either from (1a) or from (2a).

3. IRRADIATION OF A CYLINDER

The source lies on the axis of the cylinder; in essence we again have a layer, but this time one bounded laterally by cylindrical surfaces. We consider the most general case.

Problem 3. A cylinder of radius r and height h is irradiated by a point source lying axially a distance f away from the nearer face. The attenuation coefficients within and without the cylinder are μ and μ_1 respectively. Five distinct cases occur, depending how R, f, h and r are related.

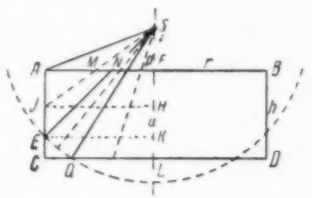

FIG. 4

(*A*) $R^2>(f+h)^2+r^2$ (all particles pass through the far end);

(*B*) $(f+h)^2+r^2>R^2>(f+h)^2$ and $R^2>f^2+r^2$ (only some particles pass through the far end);

(*C*) $(f+h)^2>R^2>f^2+r^2$ (all particles pass through the near end, but no particles pass through the far end);

(*D*) $f^2+r^2>R^2>(f+h)^2$ (all particles pass through the near end, but not all particles pass through the far end, and the side faces are quite inaccessible);

(*E*) $f^2+r^2>R^2>f^2$ (all particles are brought to rest in the cylinder).

Case B is the most complicated, and we begin with this (Fig. 4). The body is divided into three zones, (a) *ASE*, in which the particles emerge from the side faces, (b) *ESG*, in which the particles are brought to rest inside the cylinder, and (c) *GSK*, in which the particles pass through both end faces. To find the dose for zone 1, we first determine the integration limits for φ. The lower limit is $\sin^{-1}(r/R)$ (from triangle *ESK*), and the upper is $\tan^{-1}(r/f)$ (from triangle *ASF*); for the second integral along the track) the lower limit is $f/\cos\varphi$ (from triangle *SMF*) and the upper $r/\sin\varphi$.

$$Di_1^S = A \int_{\sin^{-1} r/R}^{\tan^{-1} r/f} \frac{\sin\varphi}{2} \int_{f/\cos\varphi}^{r/\sin\varphi} e^{-\mu(y-f/\cos\varphi)}\ e^{-\mu_1 f/\cos\varphi}\, dy d\varphi. \qquad N$$

For brevity we use the symbols

$$\sqrt{x^2-f^2} = s = MF;\ \sqrt{r^2+f^2} = t = SA;\ \sqrt{R^2-r^2} = u = SK.$$

$$Di_1^S = A\frac{f}{2\mu}\int_{Rf/u}^{t} \frac{1}{x^2}(1-e^{-\mu x(r-s)/s})\, e^{-\mu_1 x} dx. \qquad (c)$$

$$Di_1^S = A\,\frac{f}{2}\left\{\frac{r}{f}\left(\tan^{-1}\frac{r}{f} - \sin^{-1}\frac{r}{R}\right) + ln\frac{Rf}{tu}\right.$$
$$+\mu\left[\frac{Rf}{2u} - \frac{t}{2} + \frac{r^2}{2f}\,ln\frac{(t+f)(R-u)}{r^2} + rln\frac{(t+r)\,u}{(R+r)f}\right]$$
$$+\mu_1\left[-\frac{Rf}{u} + t - rln\frac{(t+r)\,u}{(R+r)f}\right] + \mu^2\left[\frac{r^2}{4} + \frac{r^2f^2}{12u^2} + \frac{r^2u}{6f} - \frac{r^2f}{2u} + \frac{r^2}{2}\,ln\frac{u}{f}\right]$$
$$\left.+\mu_1^2\left[\frac{r^2f^2}{4u_2} + \frac{r^2}{4} - \frac{r^2f}{2u}\right] + \mu\mu_1\left[-\frac{3r^2}{4} - \frac{r^2f^2}{4u^2} - \frac{r^2f}{u} - \frac{r^2}{2}\,ln\frac{u}{f}\right]\right\}. \qquad O$$

The integral dose for zone 2 is found from a similar double integral with different limits

$$Di_{11}^S = A \int_{\cos^{-1}(f+h)/R}^{\sin^{-1} r/R} \frac{\sin\varphi}{2} \int_{f/\cos\varphi}^{R} e^{-\mu(y-f/\cos\varphi)}\ e^{-\mu_1 f/\cos\varphi}\, dy d\varphi$$
$$= A\frac{f}{2\mu}\int_{Rf/(f+h)}^{Rf/u} \frac{1}{x^2}(1-e^{-\mu(R-x)})\ e^{-\mu_1 x}\, dx. \qquad (d)$$

$$Di_{11}^S = A\,\frac{f}{2}\left\{\frac{f+h-u}{f} - ln\frac{f+h}{u} + \mu\left[\frac{R}{2f}(u-f-h) - \frac{Rf}{2}\left(\frac{1}{u} - \frac{1}{f+h}\right)\right.\right.$$
$$\left.+ Rln\frac{f+h}{u}\right] + \mu_1\left[Rf\left(\frac{1}{u} - \frac{1}{f+h}\right) - Rln\frac{f+h}{u}\right] + \mu^2\left[-\frac{R^2}{6f}(u-f-h)\right.$$
$$+\frac{R^2f}{2}\left(\frac{1}{u} - \frac{1}{f+h}\right) - \frac{R^2f^2}{12}\left(\frac{1}{u^2} - \frac{1}{(f+h)^2}\right) - \frac{R^2}{2}\,ln\frac{f+h}{u}\Big] + \mu_1^2\left[\frac{R^2f}{2}\left(\frac{1}{u} - \frac{1}{f+h}\right)\right.$$
$$\left.-\frac{R^2f^2}{4}\left(\frac{1}{u^2} - \frac{1}{(f+h)^2}\right)\right] + \mu\mu_1\left[-R^2f\left(\frac{1}{u} - \frac{1}{f+h}\right)\right.$$
$$\left.\left.+\frac{R^2f^2}{4}\left(\frac{1}{u^2} - \frac{1}{(f+h)^2}\right) + \frac{R^2}{2}\,ln\frac{f+h}{u}\right]\right\}. \qquad P$$

For zone 3 we have

$$Di^S_{III} = A \int_0^{\cos^{-1}(f+h)/R} \frac{\sin\varphi}{2} \int_{f/\cos\varphi}^{(f+h)/\cos\varphi} e^{-\mu(y-f/\cos\varphi)}\ e^{-\mu_1 f/\cos\varphi}\, dy d\varphi =$$

$$= A\frac{f}{2\mu} \int_f^{Rf/(f+h)} \frac{1}{x^2}(1 - e^{-\mu x h/f})\, e^{-\mu_1 x} dx. \qquad \text{(e)}$$

$$Di^S_{III} = A\frac{f}{2}\left\{\frac{h}{f} ln\frac{R}{f+h} + \mu\left[-\frac{h^2}{2f}\left(\frac{R}{f+h} - 1\right)\right] + \mu_1\left[-h\left(\frac{R}{f+h} - 1\right)\right] + \right.$$

$$\left. + \mu^2\left[\frac{h^3}{12f}\left(\frac{R^2}{(f+h)^2} - 1\right)\right] + \mu_1^2\left[\frac{fh}{4}\left(\frac{R^2}{(f+h)^2} - 1\right)\right] + \mu\mu_1\left[\frac{h^2}{4}\left(\frac{R^2}{(f+h)^2} - 1\right)\right]\right\}. \quad \text{Q}$$

The total dose is found by summation as

$$Di^S_{C.B} = A\frac{f}{2}\left\{\frac{f+h-u}{f} + \frac{r}{f}\left(\tan^{-1}\frac{r}{f} - \sin^{-1}\frac{r}{R}\right) + ln\frac{f}{t} - \frac{f+h}{f} ln\frac{f+h}{R} + \right.$$

$$+ \mu\left[-\frac{t}{2} + \frac{Ru}{2f} + \frac{h^2}{2f} - \frac{Rh}{f} + \frac{r^2}{2f} ln\frac{(t+f)(R-u)}{r^2} + r ln\frac{(t+r)u}{(R+r)f} + R ln\frac{f+h}{u}\right] +$$

$$+ \mu_1\left[t - h - R - r ln\frac{(t+r)u}{(R+r)f} - R ln\frac{f+h}{u}\right] + \mu^2\left[\frac{r^2}{4} - \frac{f^2}{12} - \frac{u^3}{6f} + \frac{fu}{2} - \right.$$

$$- \frac{h^3}{12f} + \frac{R^2h^2}{4f(f+h)} + \frac{r^2}{2} ln\frac{u}{f} - \frac{R^2}{2} ln\frac{f+h}{u}\Big] + \mu_1^2\left[\frac{r^2}{4} - \frac{f^2}{4} + \frac{fu}{4} - \frac{R^2f}{4(f+h)}\right] +$$

$$\left. + \mu\mu_1\left[-\frac{3r^2}{4} + \frac{f^2}{4} - fu - \frac{h^2}{4} + \frac{R^2}{4} + \frac{R^2f}{2(f+h)} + \frac{r^2}{2} ln\frac{f}{u} + \frac{R^2}{2} ln\frac{f+h}{u}\right]\right\}. \qquad (3)$$

When $f=0$ all terms in μ_1 drop out and the formulae simplify to

$$Di^S_{C.B.} = A\frac{1}{2}\left\{h - u + r\left(\frac{\pi}{2} - \sin^{-1}\frac{r}{R}\right) + hln\frac{R}{h} + \mu\left[\frac{Ru}{2} + \frac{h^2}{2} - \right.\right.$$

$$\left.\left. - Rh + \frac{r^2}{2} ln\frac{R-u}{r}\right] + \mu^2\left[-\frac{u^3}{6} - \frac{h^3}{12} + \frac{R^2h}{4}\right]\right\}. \qquad (3a)$$

If we put $\mu=\mu_1=0$ then

$$Di^S_{C.B.} = A\frac{1}{2}\left\{f + h - u + r\left(\tan^{-1}\frac{r}{f} - \sin^{-1}\frac{r}{R}\right) + fln\frac{f}{t}\right.$$

$$\left. - (f+h) ln\frac{f+h}{R}\right\}. \qquad (3b)$$

If we put $\mu=\mu_1\neq 0$ then

$$Di^S_{C.B.} = A\frac{f}{2}\left\{\frac{f+h-u}{f} + \frac{r}{f}\left(\tan^{-1}\frac{r}{f} - \sin^{-1}\frac{r}{R}\right) + ln\frac{f}{t}\right.$$

$$- \frac{f+h}{f} ln\frac{f+h}{R} + \mu\left[\frac{t}{2} + h - \frac{R(f+h)}{f} + \frac{h^2}{2f} + \frac{Ru}{2f}\right.$$

$$\left.\left. + \frac{r^2}{2f} ln\frac{(t+f)(R-u)}{r^2}\right] + \mu^2\left[-\frac{r^2}{4} - \frac{u^3}{6f} - \frac{(f+h)^3}{12f} + \frac{R^2(f+h)}{4f}\right]\right. \qquad (3c)$$

If μ and $\mu_1 \gg 1$, (3) cannot be used, because the series diverges, so asymptotically convergent series obtained by integration by parts with μ and μ_1 entering to negative powers must be used. The expressions to be used are then (c), (d) and (e).

For zone 1:

$$Di_{\mathrm{I}}^{S} = A\frac{f}{2\mu}\left\{\int_{Rf/u}^{t}\frac{1}{x^2}e^{-\mu_1 x}\,dx - \int_{Rf/u}^{t}\frac{1}{x^2}e^{-\mu x r/s-(\mu_1-\mu)x}\,dx\right\}$$

$$= A\frac{f}{2\mu}\left\{\mu_1\Big|_{\mu_1 t}^{\mu_1 Rf/u}\left[\frac{e^{-z}}{z}+Ei\,(-z)\right]\cdot\right. \qquad \text{R}$$

$$\left.-\int_{Rf/u}^{t}\frac{s^3}{x^2\,[\mu r f^2-(\mu_1-\mu)\,s^3]}\,e^{-\mu r x/s-(\mu_1-\mu)x}\left[1-\frac{s}{x\,[\mu r f^2-(\mu_1-\mu)\,s^3]^{\frac{1}{2}}}\right]\right\}.$$

For zone 2:

$$Di_{\mathrm{II}}^{S} = A\frac{f}{2\mu}\left\{\int_{Rf/(f+h)}^{Rf/u}\frac{1}{x^3}e^{-\mu_1 x}\,dx - \int_{Rf/(f+h)}^{Rf/u}\frac{1}{x^2}e^{-\mu R}\;e^{-(\mu_1-\mu)x}\,dx\right. \qquad \text{S}$$

$$= A\frac{f}{2\mu}\left\{\mu_1\Big|_{\mu_1 Rf/u}^{\mu_1 Rf/(f+h)}\left[\frac{e^{-z}}{z}+Ei\,(-z)\right]-(\mu_1-\mu)\,e^{-\mu R}\Big|_{(\mu_1-\mu)Rf/u}^{(\mu_1-\mu)Rf/(f+h)}\left[\frac{e^{-z}}{z}\right.\right.$$

$$\left.\left.+\,Ei\,(-z)\right]\right\}.$$

For zone 3:

$$Di_{\mathrm{III}}^{S} = A\frac{f}{2\mu}\left\{\mu_1\Big|_{\mu_1 Rf/(f+h)}^{\mu_1 f}\left[\frac{e^{-z}}{z}+Ei\,(-z)\right]-\frac{(\mu h+\mu_1 f)}{f+h}\Big|_{(\mu h+\mu f)R/(f+h)}^{\mu h+\mu_1 f}\left[\frac{e^{-z}}{z}\right.\right. \qquad \text{T}$$

$$\left.\left.+\,Ei(-z)\right]\right\}.$$

For the cylinders as a whole:

$$Di_{\mathrm{C.B.}}^{S} = A\frac{f}{2\mu}\left\{\mu_1\Big|_{\mu_1 t}^{\mu_1 f}\left[\frac{e^{-z}}{z}+Ei\,(-z)\right]-(\mu_1-\mu)\,e^{-\mu R}\Big|_{(\mu_1-\mu)Rf/u}^{(\mu_1-\mu)Rf/(f+h)}\left[\frac{e^{-z}}{z}\right.\right.$$

$$\left.+\,Ei\,(-z)\right]-\frac{\mu h+\mu_1 f}{f+h}\Big|_{(\mu h+\mu_1 f)R/(f+h)}^{\mu h+\mu_1 f}\left[\frac{e^{-z}}{z}+Ei\,(-z)\right]$$

$$\left.-\int_{Rf/u}^{t}\frac{s^3 e^{-\mu r x/s-(\mu_1-\mu)x}}{x^2\,[\mu r f^2-(\mu_1-\mu)\,s^3]}\left[1-\frac{s}{x\,[\mu r f^2-(\mu_1-\mu)\,s^3]^{\frac{1}{2}}}\right]\right\}. \qquad (4)$$

Case A (Fig. 5), where $R^2>(f+h)^2+r^2$, is much simpler than case *B*, since only

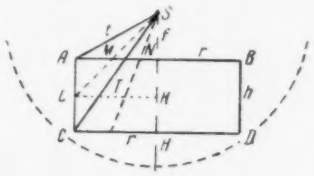

FIG. 5

two zones occur, namely ASC, where the particles escape from the side surface, and CSK, where the particles pass through both end faces. The range does not enter into the calculations. The total dose is found by summing for these two zones, and is

$$Di^S_{C.A} = A\frac{f}{2}\left\{\frac{r}{f}\left(\tan^{-1}\frac{r}{s} - \tan^{-1}\frac{r}{f+h}\right) - ln\frac{t}{f} - \frac{f+h}{f}ln\frac{f+h}{T}\right.$$
$$+\mu\left[-\frac{t}{2}+\frac{h^2}{2f}+\frac{T(f-h)}{2f}+\frac{r^2}{2f}ln\frac{(t+f)(T-f-h)}{r^2}+r^2ln\frac{(t+r)(f+h)}{(T+r)f}\right]$$
$$+\mu_1\left[t+h-T-rln\frac{(t+r)(f+h)}{(T+r)f}\right]+\mu^2\left[\frac{r^2h}{4(f+h)}+\frac{r^2h}{4f}+\frac{r^2}{2}ln\frac{f+h}{f}\right]$$
$$\left.+\mu_1^2\left[\frac{r^2h}{4(f+h)}\right]+\mu\mu_1\left[-\frac{r^2h}{2(f+h)}\right]\right\}, \tag{5}$$

where

$$T=\sqrt{(f+h)^2+r^2}=SC.$$

If we put $f=0$, then we have

$$Di^S_{C.A} = A\frac{1}{2}\left\{r\tan^{-1}\cdot\frac{h}{r}+hln\frac{\sqrt{h^2+r^2}}{h}+\mu\left[\frac{h^2}{2}-\frac{h\sqrt{h^2+r^2}}{2}\cdot\right.\right.$$
$$\left.\left.+\frac{r^2}{2}ln\frac{\sqrt{h^2+r^2}-h}{2}\right]+\mu^2\left[\frac{r^2h}{4}\right]\right\}. \tag{5a}$$

If we put $\mu=\mu_1=0$ then

$$Di^S_{C.A} = A\frac{1}{2}\left\{r\left(\tan^{-1}\frac{r}{f}-\tan^{-1}\cdot\frac{r}{f+h}\right)-fln\frac{\sqrt{h^2+r^2}}{f}\right.$$
$$\left.+(f+h)ln\frac{\sqrt{(f+h)^2+r^2}}{f+h}\right. . \tag{5b}$$

If we put $\mu=\mu_1\neq 0$ then

$$Di^S_{C.A} = A\frac{1}{2}\left\{r\left(\tan^{-1}\frac{r}{f}-\tan^{-1}\frac{r}{f+h}\right)-fln\frac{\sqrt{h^2+r^2}}{f}\right.$$
$$+(f+h)ln\frac{\sqrt{(f+h)^2+r^2}}{f+h}+\mu\left[\frac{(f+h)^2}{2}+\frac{f(t-f)}{2}-\frac{\sqrt{(f+h)^2+r^2}}{2}(f+h)\right.$$
$$\left.\left.+\frac{r^2}{2}ln\frac{(t+f)[\sqrt{(f+h)^2+r^2}-(f+h)]}{r^2}\right]+\mu^2\left[\frac{r^2h}{4}+\frac{r^2f}{2}ln\frac{f+h}{f}\right]\right\}. \tag{5c}$$

Case C (Fig. 6), where $(f+h)^2>R^2>f^2+r^2$ has the particles either escaping from the sides, or stopping in the cylinder. The range does not enter into the calculations. There are two zones, AES and EGS.

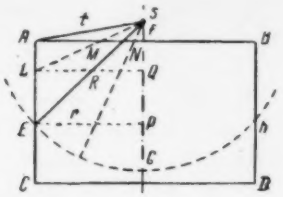

FIG. 6

$$Di^{S}_{C.C} = A\frac{1}{2}\left\{r\left(\cos^{-1}\frac{f}{t} - \cos^{-1}\frac{u}{R}\right)\right.$$
$$+ R - u - f\ln\frac{t}{f}$$
$$- \mu\left[\frac{R(R-u)}{2} + \frac{f(t-f)}{2} - \frac{r^2}{2}\ln\frac{(t+f)(R-u)}{r^2}\right.$$
$$\left. - rf\ln\frac{(t+r)u}{(R+r)f} - Rf\ln\frac{R}{u}\right] - \mu_1\left[-f(t-f) + rf\ln\frac{(t+r)u}{(R+u)f} + Rf\ln\frac{R}{u}\right]$$
$$+\mu^2\left[\frac{R^3 - u^3}{6} - \frac{f^2(R-u)}{2} + \frac{r^2f}{4} + \frac{r^2f}{2}\ln\frac{u}{f} - \frac{R^2f}{2}\ln\frac{R}{u}\right] + \mu_1^2\left[\frac{f^2(R-u)}{2} + \frac{r^2f}{4}\right]$$
$$\left. + \mu\mu_1\left[f^2(R-u) - \frac{3r^2f}{4} + \frac{R^2f}{2}\ln\frac{R}{u} + \frac{r^2f}{2}\ln\frac{u}{f}\right]\right\}. \qquad (6)$$

If the source lies at the centre of one end face

$$Di^{S}_{C.C} = A\frac{1}{2}\left\{R - u + r\cos^{-1}\frac{r}{R} - \mu\left[\frac{R(R-u)}{2} - \frac{r^2}{2}\ln\frac{R-u}{r}\right]\right.$$
$$\left. + \mu^2\left[\frac{R^3 - u^3}{6}\right]\right\}. \qquad (6a)$$

Cases D and *E* do not need to be considered, since they are the same as the "thin" and "thick" layers above.

4. IRRADIATION OF A SPHERE

The problems here are again divided according to how R, r and ρ (the distance from the source to the centre of the sphere) are related. When $\rho > r$ we have three cases. In *A* all particles entering the sphere stop within it; in *B* some particles pass through, and in *C* all pass through.

Problem 4. A sphere of radius r is irradiated by a point source S emitting particles of range R a distance ρ from the centre of the sphere.

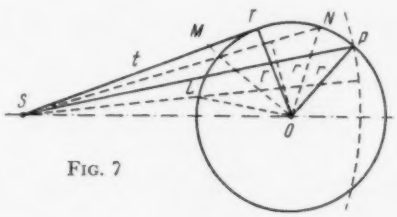

FIG. 7

Case B. $\sqrt{\rho^2-r^2}<R<\rho+r$. Here $\sqrt{\rho^2-r^2}=t$ is the length of the tangent from S (Fig. 7). The irradiated space is divided into zone 1, where all particles entering the sphere stop within it; this is defined by the angle $\mathrm{PSO}=\theta_1$, which can be expressed parametrically by using triangle PSO. We have $r^2=R^2+\rho^2-2R\rho\cos\theta_1$, so $\theta_1=\cos^{-1}(R^2+t^2)/2R\rho$, where $t^2=\rho^2-r^2$. This is also the upper limit to the integral with respect to φ. The lower limit for the integral with respect to y is found from triangle SLO.

$$r^2=\rho^2+SL^2-2\rho\cdot SL\cos\varphi;\ SL^2-2\rho\cos\varphi\cdot SL+t^2=0;$$
$$SL=\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2}. \qquad \text{V}$$

The upper limit is R, the range

$$Di_1^S=A\int_0^{\cos^{-1}(R^2+t^2)/2R\rho}\frac{\sin\varphi}{2}\int_{\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2}}^{R}e^{-\mu(y-\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2})}\,e^{-\mu_1(\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2})}\,dy\,d\varphi. \qquad \text{W}$$

We now put $\rho\cos\varphi=x$ and $\sqrt{x^2-t^2}=s$. Integration with respect to y gives

$$Di_1^S=\frac{A}{2\rho\mu}\int_{\rho}^{(R^2+t^2)/2R}(e^{-\mu(R-x+s)}-1)e^{-\mu_1(x-s)}\,dx. \qquad \text{(f)}$$

Term by term integration, substitution and algebraic manipulation give the integral dose for zone 1 as

$$\begin{aligned}Di_1^S=A\frac{1}{2\rho}\Big\{&\frac{t^4}{4R^2}-\frac{(R-\rho)^2}{2}-\frac{(\rho-r)^2}{4}+\frac{r^2}{2}+\frac{t^2}{2}\ln\frac{R}{\rho+r}\\
&+\mu\Big[\frac{R^3}{4}+\frac{t^6}{12R^3}+\frac{(R^2-t^2)(t^2-2R\rho)}{4R}-\frac{\rho^3-r^3}{3}-\frac{t^2(R^2+t^2)}{4R}\\
&+\frac{R\rho(\rho-2)}{2}-\frac{Rt^2}{2}\ln\frac{R}{\rho+r}\Big]+\mu_1\Big[-\frac{t^6}{6R^3}+\frac{t^2(t^2-2R\rho)}{2R}\\
&+\frac{2}{3}(\rho^3-r^3)+\frac{t^2(R^2+t^2)}{4R}-\frac{R\rho(\rho-r)}{2}+\frac{Rt^2}{2}\ln\frac{R}{\rho+r}\Big]\\
&+\mu^2\Big[-\frac{R^2(R^2+t^2)}{12}+\frac{t^4(3R^2-t^2)}{48R^4}+\frac{R^3\rho}{6}-\\
&-\frac{(2R-\rho)(\rho-r)^2(\rho+2r)}{12}+\frac{t^2(R^2+t^2)}{8}-\frac{R^2\rho(\rho-r)}{4}\\
&+\frac{R^2t^2}{4}\ln\frac{R}{\rho+r}\Big]=\mu_1^2\Big[\frac{t^4(3R^2-t^2)^2}{48R^4}-\\
&-\frac{2t^4(R^2+t^2)(3R^2-t^2)}{48R^4}-\frac{(2R-3\rho)(\rho-r)^2(\rho+2r)}{12}\Big]\\
&+\mu\mu_1\Big[-\frac{2t^4(3R^2-t^2)^2}{48R^4}+\frac{t^4(R^2+t^2)(3R^2-t^2)}{48R^4}+\frac{(4R-3\rho)(\rho-r)^2(\rho+2r)}{12}\\
&-\frac{t^2(R^2+t^2)}{8}+\frac{R^2\rho(\rho-r)}{4}-\frac{R^2t^2}{4}\ln\frac{R}{\rho+r}\Big]\Big\}. \qquad \text{X}\end{aligned}$$

To get the integral dose for zone 2 the limits to the integral with respect to φ are $\cos^{-1}(R^2+t^2)/2R\rho$ and $\cos^{-1}t/\rho$. The limits for the integral with respect to y are the roots of a quadratic equation deriving from triangle SNO:

$$y \text{ 1 and } 2 = \rho\cos\varphi \mp \sqrt{\rho^2\cos^2\varphi - t^2}$$

$$Di^s_{II} = A \int_{\cos^{-1}(R^2+t^2)/2R\rho}^{\cos^{-1} t/\rho} \frac{\sin\varphi}{2} \int_{\rho\cos\varphi - \sqrt{\rho^2\cos^2\varphi - t^2}}^{\rho\cos\varphi + \sqrt{\rho^2\cos^2\varphi - t^2}} e^{-\mu(y - \rho\cos\varphi\sqrt{\rho^2\cos^2\varphi - t^2})} \quad \text{Y}$$

$$e^{-\mu_1(\rho\cos\varphi - \sqrt{\rho^2\cos^2\varphi - t^2})}\, dy d\varphi.$$

Summing all terms gives

$$Di^s_{II} = A\,\frac{1}{2\rho}\left\{\frac{R^4 - t^4}{4R^2} - t^2 \ln\frac{R}{t} - \mu\left[\frac{(R-t)^4(R^2+4Rt+t^2)}{12R^3}\right]\right.$$

$$- \mu_1\left[\frac{(R-t)^3 t^2(3R+t)}{6R^3}\right] + \mu^2\left[\frac{(R^4-t^4)(R^4-8R^2t^2+t^4)}{48R^4} + \frac{t^4}{2}\ln\frac{R}{t}\right] \quad \text{Z}$$

$$+ \mu_1^2\left[\frac{t^4(R^2-t^2)(t^2-3R^2)}{16R^4} + \frac{t^4}{4}\ln\frac{R}{t}\right] + \mu\mu_1\left[\frac{t^2(R^2-t^2)(2R^4+5R^2t^2-t^4)}{16R^4}\right.$$

$$\left.\left. - \frac{3t^4}{4}\ln\frac{R}{t}\right]\right\}.$$

Adding the integral doses for the zones we get the total as

$$Di^S_{S.B} = A\,\frac{1}{2\rho}\left[\frac{R^2}{4} + \frac{r^2}{2} - \frac{(R-\rho)^2}{2} - \frac{(\rho-r)^2}{4} - \frac{t^2}{2}\ln\frac{R}{\rho-r} + \mu\left[\frac{R^3}{6} + \frac{(R^2+t^2)^2}{4R}\right.\right.$$

$$- \frac{(R^2-t^2)(R+2\rho)}{4} - \frac{\rho^3-r^3}{3} - \frac{4t^3}{3} + \frac{R\rho(\rho-r)}{2} - \frac{Rt^2}{2}$$

$$\ln\frac{R}{\rho+r}\Big] + \mu_1\Big[-\frac{t^2(R^2+t^2)}{4R} + \frac{2(\rho-r)^3}{3} - \rho(\rho-r)^2$$

$$+ \frac{4t^3}{3} - \frac{R\rho(\rho-r)}{2} + \frac{Rt^2}{2}\ln\frac{R}{\rho+r}\Big]$$

$$+ \mu^2\left[\frac{t^6}{24R^2} + \frac{5t^4}{16} - \frac{(2R-\rho)(\rho-r)^2(\rho+2r)}{12} + \frac{R^3\rho}{6} - \frac{R^2(R^2+2t^2)}{16}\right.$$

$$- \frac{R^2\rho(\rho-r)}{4} + \frac{t^4}{2}\ln\frac{R}{t} + \frac{R^2t^2}{4}\ln\frac{R}{\rho+r}\Big] + \mu_1^2\Big[\frac{t^6}{24R^2} - \frac{t^4}{8}$$

$$- \frac{(2R-3\rho)(\rho-r)^2(\rho+2r)}{12} + \frac{t^4}{4}\ln\frac{R}{t}\Big] + \mu\mu_1\Big[-\frac{t^6}{12R^2} - \frac{t^4}{4}$$

$$\left.\left. + \frac{(4R-3\rho)(\rho-r)^2(\rho+2r)}{12} + \frac{R^2(\rho-r)\rho}{4} - \frac{3t^4}{4}\ln\frac{R}{t} - \frac{R^2t^2}{4}\ln\frac{R}{\rho+r}\right]\right\}. \quad (7)$$

If we neglect the absorption and retain R finite we get a formula essentially distinct from Souttar's [3] for radon, because R has been retained as finite.

If $\mu=\mu_1=0$ we get

$$Di^S_{S.B} = A\,\frac{1}{2\rho}\left[\frac{R^2}{4} + \frac{r^2}{2} - \frac{(R-\rho)^2}{2} - \frac{(\rho-r)^2}{4} - \frac{t^2}{2}\ln\frac{R}{\rho-r}\right]. \quad (7a)$$

If $\mu=\mu_1\neq 0$ we get

$$Di^S_{S.B} = A\,\frac{1}{2\rho}\left[\frac{R^2}{4} + \frac{r^2}{2} - \frac{(R-\rho)^2}{2} - \frac{(\rho-r)^2}{4} - \frac{t^2}{2}\ln\frac{R}{\rho-r}\right] + \quad (7b)$$

$$+\mu\left[\frac{(R-\rho)^3}{6} - \frac{r^2(R-\rho)}{2} - \frac{r^3}{3}\right] + \mu^2\left[-\frac{(R^2+t^2)^2}{16} + \frac{\rho}{6}(R^3-\rho^3+r^3) + \frac{\rho^2t^2}{4}\right]\Big\}.$$

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If the source lies on the surface of the sphere, i.e. if $\rho=r$ and $t=0$, we get

$$Di^S_{S.B} = A\frac{1}{2\rho}\left\{R\left(r-\frac{R}{4}\right)+\mu\left[\frac{R^2}{2}\left(\frac{R}{3}-r\right)\right]-\mu^2\left[\frac{R^3}{2}\left(\frac{r}{3}-\frac{R}{8}\right)\right]\right\}. \qquad (7c)$$

We now apply to (f) the other approximate integration method, applicable for μ and $\mu_1 \gg 1$.

Zone 1:

$$Di^S_I = A\frac{1}{2\rho\mu}\left[\int_{\rho}^{(R^2+t^2)/2R} e^{-\mu R}e^{-(\mu_1-\mu)(x-s)}\,dx - \int_{\rho}^{(R^2+t^2)/2R} e^{-\mu_1(x-s)}\,dx\right], \qquad A^1$$

where

$$s=\sqrt{x^2-t^2}, \text{ and } t=\sqrt{\rho^2-r^2}. \qquad B^1$$

Zone 2:

$$Di^S_{II} = A\frac{1}{2\rho\mu}\int_{t}^{(R^2+t^2)/2R} e^{-\mu_1(x-s)}\,dx - \int_{t}^{(R^2+t^2)/2R} e^{-\mu_1(x-s)-2\mu s}\,dx \qquad C^1$$

Adding the integral doses for the zones we get the total as

$$Di^S_{S.B} = A\frac{1}{2\rho\mu}\left\{\int_{\rho}^{(R^2+t^2)/2R}\left[\frac{e^{-\mu R}}{\mu_1-\mu}\,\frac{1}{x-s}\,e^{-(\mu_1-\mu)(x-s)} - \frac{1}{\mu_1}\,\frac{s}{x-s}\,e^{-\mu_1(x-s)} - \right.\right.$$
$$\left. - \frac{t^2e^{-\mu R}}{(\mu_1-\mu)^2}e^{-(\mu_1-\mu)(x-s)} + \frac{t^2}{\mu_1^2}\frac{1}{(x-s)^3}e^{-\mu_1(x-s)}\right] +$$
$$+ \int_{t}^{(R^2+t^2)/2R}\left[\frac{s}{\mu_1(x-s)}e^{-\mu_1(x-s)} - \frac{s}{\mu_1(x-s)-2\mu s}e^{-\mu_1(x-s)-2\mu s} - \right.$$
$$\left.\left. - \frac{t^2}{\mu_1^2}\,\frac{1}{(x-s)^3}e^{-\mu_1(x-s)} - \frac{t^2(\mu_1-2\mu)}{[\mu_1(x-s)-2\mu s]^3}e^{-\mu_1(x-s)-2\mu s}\right]\right\}. \qquad (7')$$

Case A. All particles come to rest in the sphere, and we have one zone only $(\rho-r)<R<t$ (Fig. 8). The limits to the integrals are deduced as for case *B*. We

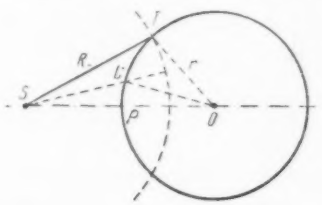

FIG. 8

retain the notation $s=\sqrt{x^2-t^2}$ and $t=\sqrt{\rho^2-r^2}$.

$$Di^{S}_{S.A} = A \int_0^{\cos^{-1}(R^2+t^2)/2R\rho} \frac{\sin\varphi}{2} \int_{\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2}}^{R} e^{-\mu(y-\rho\cos\varphi+\sqrt{\rho^2\cos^2\varphi-t^2})} \cdot$$

$$\cdot\, e^{-\mu_1(\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2})}\, dyd\varphi = -A\frac{1}{2\rho}\int_{\rho}^{(R^2+t^2)/2R}\int_{x-s}^{R} e^{-\mu y}\, e^{\mu(x-1)}\, e^{-\mu_1(x-s)}dxdy =$$

$$= A\frac{1}{2\rho\mu}\int_{\rho}^{(R^2+t^2)/2R}(e^{-\mu(R-x+s)}-1)\,e^{-\mu_1(x-s)}dx. \qquad \mathrm{D}^1$$

Formula (f) for zone 1 of case B is again obtained, so the formulae for that case also apply to this problem. We consider only some derivative special cases.

If we have $\mu=\mu_1=0$

$$\mathrm{D}^{1S}_{S.A} = A\frac{1}{2\rho}\left\{\frac{t^4}{4R^2}-\frac{(R-\rho)^2}{2}-\frac{(\rho-r)^2}{4}+\frac{r^2}{2}+\frac{t^2}{2}\ln\frac{R}{\rho+r}\right\}. \qquad (8a)$$

If we have $\mu=\mu_1\neq 0$

$$Di^{S}_{S.A} = A\frac{1}{2\rho}\left\{\frac{t^4}{4R^2}-\frac{(R-\rho)^2}{2}-\frac{(\rho-r)^2}{4}+\frac{r^2}{2}+\frac{t^2}{2}\ln\frac{R}{\rho+r}+\right.$$

$$+\mu\left[\frac{(R^2+t^2-R\rho)^2}{4R}-\frac{R(\rho^2+t^2)}{4}+\frac{\rho^3-r^3}{3}-\frac{t^6}{12R^3}\right]+$$

$$\left.+\mu^2\left[-\frac{R^2(R^2+t^2)}{12}-\frac{t^4(R^2+t^2)(3R^2-t^2)}{48R^4}+\frac{\rho}{6}(R^3-\rho^3+r^3)+\frac{\rho^2t^2}{4}\right]\right\}. \qquad (8b)$$

Case C, where $R>\rho+r$ and all particles pass right through the sphere (Fig. 9) is

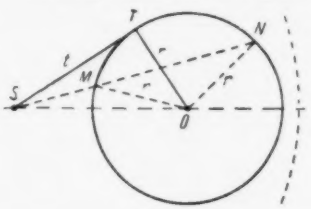

FIG. 9

solved at once, since we just apply the formula for zone 2 of case B, but with lower limits of 0 (for φ) and t (for x). The formula is

$$Di^{S}_{S.C} = A\int_0^{\cos^{-1}t/\rho}\frac{\sin\varphi}{2}\int_{\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2}}^{\rho\cos\varphi+\sqrt{\rho^2\cos^2\varphi-t^2}} e^{-\mu\left(y-\rho\cos\varphi+\sqrt{\rho^2\cos^2\varphi-t^2}\right)}\times$$

$$\times\, e^{-\mu_1(\rho\cos\varphi-\sqrt{\rho^2\cos^2\varphi-t^2})}\, dyd\varphi. \qquad \mathrm{E}^1$$

After manipulation we finally get

$$Di^{S}_{S.C} = A\frac{1}{2\rho}\left\{\rho r-\frac{t^2}{2}\ln\frac{\rho+r}{\rho-r}+\mu\left[\frac{4(\rho^3-t^3)}{3}-2\rho r^2\right]+\right.$$

$$+\mu_1\left[-\frac{4(\rho^3-t^3)}{3}+\frac{2r^2(3\rho-r)}{3}\right]+\mu^2\left[\frac{\rho r^3}{3}-\frac{\rho r(\rho^2-r^2)}{2}+\frac{t^4}{4}\ln\frac{\rho+r}{\rho-r}\right]+$$

$$+\mu_1^2\left[-\frac{r(\rho-r)^2(\rho+2r)}{4}+\frac{t^4}{8}\ln\frac{\rho+r}{\rho-r}\right]+\mu\mu_1\left[\frac{r(\rho-r)(3\rho^2+3\rho r-2r^2)}{4}-\right.$$

$$\left.\left.-\frac{3t^4}{8}\ln\frac{\rho+r}{\rho-r}\right]\right\}. \qquad (9)$$

If $\mu=\mu_1=0$ we get

$$Di^S_{S.C} = A\frac{1}{2\rho}\left\{\rho r - \frac{t^2}{2} ln\frac{\rho+r}{\rho-r}\right\}, \tag{9a}$$

i.e. just Souttar's formula.
If $\mu=\mu_1\neq 0$ we get

$$Di^S_{S.C} = A\frac{1}{2\rho}\left\{\rho r - \frac{t^2}{2} ln\frac{\rho+r}{\rho-r} - \mu\left[\frac{2r^3}{3}\right] + \mu^2\left[\frac{\rho r^3}{3}\right]\right\}. \tag{9b}$$

If the source lies within the sphere we get cases *A*, *B* and *C* in just the same way.
Case A. All particles come to rest inside the sphere (Fig. 10)

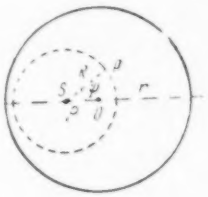

FIG. 10

and we have

$$Di^S_{S.A} = A\int_0^R e^{-\mu x}\, dx = A(1-e^{-\mu R}) \approx A\mu\left(R - \mu\frac{R^2}{2} + \mu^2\frac{R^3}{6}\right). \tag{10}$$

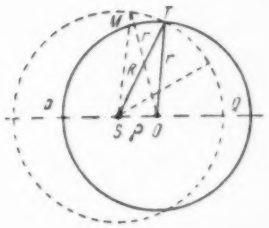

FIG. 11

Case B. The irradiated volume is divided into zones (Fig. 11). In zone 1 (*PST*) the particles escape from the sphere, while in zone 2 (*TSQ*) all particles come to rest inside the sphere. For zone 1

$$Di^S_1 = A\int_0^{\cos^{-1}(t^2-R^2)/2R\rho} \frac{\sin\varphi}{2} \int_0^{-\rho\cos\varphi+\sqrt{\rho^2\cos^2\varphi-t^2}} e^{-\mu\nu}\, dyd\varphi. \tag{F1}$$

The upper limit to the first integral is determined from triangle STO: $r^2=R^2+\rho^2+2R\rho\cos\theta_1$, where $\theta_1=P\hat{S}T$; so $\theta_1=\cos^{-1}(r^2-\rho^2-R^2)/2R\rho=\cos^{-1}(t^2-R^2)/2R\rho$ where $t=\sqrt{r^2-\rho^2}$; the lower limit is zero. The second integral has a lower limit of zero, while the upper is determined from triangle SMO using $r^2=MS^2+\rho^2+2MS\rho\cos\varphi$, where $\varphi=PSM$ ($\cos M\hat{S}O=-\cos P\hat{S}M$).

$$MS^2 + 2\rho\cos\varphi \;\; MS - t^2 = 0; \quad MS = -\rho\cos\varphi + \sqrt{\rho^2\cos^2\varphi + t^2}. \qquad G^1$$

Changing variables by the substitution $\rho\cos\varphi = x$ and using $x^2 + t^2 = q^2$ we have

$$Di_1^S = -A\frac{1}{2\rho}\int_{\rho}^{(t^2-R^2)/2R}\int_{0}^{-x+q} e^{-\mu y}\, dydx = A\frac{1}{2\rho\mu}\int_{\rho}^{(t^2-R^2)/2R}(e^{\mu(x-q)} - 1)\, dx. \qquad (g)$$

Expanding $\exp\mu(x-q)$ in series and integrating term by term, we finally get

$$Di_I^S = A\frac{1}{2\rho}\left\{\frac{R^2}{4} - \frac{(r-\rho)^2}{4} - \frac{t^2}{2}\ln\frac{r-\rho}{R} + \mu\left[-\frac{t^2(R+2\rho)}{4} - \frac{R^3}{12} + \frac{r^3-\rho^3}{3}\right] + \mu^2\left[\frac{t^4}{16} + \frac{R^4 - R^2t^2}{48} + \frac{\rho(r^3-\rho^3)}{6}\right]\right\}. \qquad H^1$$

For zone 2

$$Di_{II}^S = A_{\cos^{-1}}\int_{(t^2-R^2)/2R\rho}^{\pi}\frac{\sin\varphi}{2}\int_0^R e^{-\mu y}\, dyd\varphi \qquad I^1$$

$$= A\frac{1}{2\rho}\left\{\frac{r^2-(R-\rho)^2}{2} - \mu R\frac{r^2-(R-\rho)^2}{4} + \mu^2R^2\frac{r^2-(R-\rho)^2}{12}\right\}.$$

Summation for the two zones gives the total dose for case B as

$$Di_{S.B}^S = A\frac{1}{2\rho}\left\{\frac{R^2}{4} + \frac{r^2}{2} - \frac{(R-\rho)^2}{2} - \frac{(r-\rho)^2}{4} - \frac{t^2}{2}\ln\frac{r-\rho}{R} - \mu\left[\frac{3(R+\rho)t^2}{2} + \frac{R^2(3\rho-R)}{2} - \frac{r^3-\rho^3}{3}\right] + \mu^2\left[\frac{t^4}{16} - \frac{R^2(R^2-t^2)}{16} + \frac{\rho}{6}(R^3 + r^3 - \rho^3)\right]\right\}. \qquad (11)$$

If $\mu = \mu_1 = 0$ we get

$$Di_{S.B}^S = A\frac{1}{2\rho}\left\{\frac{R^2-(r-\rho)^2}{4} + \frac{r^2-(R-\rho)^2}{2} - \frac{t^2}{2}\ln\frac{r-\rho}{R}\right\}. \qquad (11a)$$

If $\mu \gg 1$ we have to use another method of approximate integration, using formula (g). Summation for the two zones gives the total dose for case B as

$$Di_{S.B}^S = A\frac{1}{2\rho\mu}\int_{\rho}^{(t^2-R^2)/2R}\left\{\frac{1}{\mu}\frac{q}{q-x}e^{-\mu(q-x)} - \frac{1}{\mu^2}\frac{q+x}{(q-x)^2}e^{-\mu(q-x)} + \frac{1}{\mu^2}\frac{r^2-(R-\rho)^2}{2R}e^{-\mu R}\right\}. \qquad I^{11}$$

Case C is solved in one way, and corresponds to a sphere irradiated by rays of unlimited range (Fig. 12). The limits for the first integration are 0 and π, while for

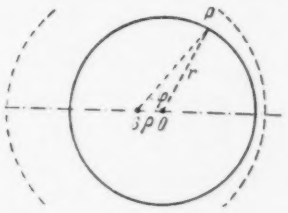

FIG. 12

the second the lower limit is 0, while the upper is found from triangle SPO

$$r^2 = SP^2 + \rho^2 - 2SP\rho\cos\varphi, \qquad J^1$$

so we have

$$SP = \rho\cos\varphi + \sqrt{\rho^2\cos^2\varphi + t^2}, \qquad K^1$$

where $t = \sqrt{r^2-\rho^2}$.

$$Di^S_{S.C} = A\int_0^{\pi}\frac{\sin\varphi}{2}\int_0^{\rho\cos\varphi+\sqrt{\rho^2\cos^2\varphi+t^2}} e^{-\mu\nu}dyd\varphi$$

$$= A\,\frac{1}{2\rho}\left\{\rho r + \frac{t^2}{2}\ln\frac{r+\rho}{r-\rho} - \mu\left[\frac{2(\rho^3+r^3)}{3} + \rho t^2\right] + \mu^2\left[\frac{\rho r^3}{3}\right]\right\}. \qquad (12)$$

If $\mu=\mu_1=0$ we get

$$Di^S_{S.C} = A\frac{1}{2\rho}\left\{\rho r + \frac{t^2}{2}\ln\frac{r+\rho}{r-\rho}\right\}. \qquad (12a)$$

This is easily transformed to Souttar's formula

$$Di^S_{S.C} = A\left(\frac{r}{2} + \frac{\rho^2-r^2}{4\rho}\ln\frac{r+\rho}{r-\rho}\right). \qquad L^1$$

If the source lies at the centre of the sphere ($\rho=0$), then $Di^s_{s.c}=Ar/2$.

The difficulties encountered if the differences in range in the two media have to be allowed for can be reduced by assuming that the medium outside the body is of very low stopping power (e.g. air), so virtually all the energy reaches the body. The range can then be taken as constant at R. We consider how this affects the integral dose to a sphere when $R<2r$ (Fig. 13). There are two zones, zone 1 in which the

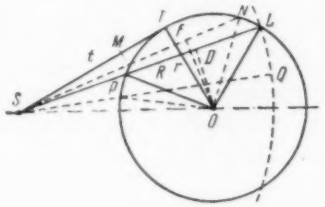

FIG. 13

particles come to rest inside the sphere, and zone 2 where they pass right through. The boundary to zone 1 is defined by the angle θ_1, while the second zone lies between θ_1 and θ_2. θ_1 is determined from triangle SDO, which gives $DO=SO\sin\theta_1$; $\theta_1=\sin^{-1}(DO/\rho)$; triangle DOL gives $DO^2=LO^2-LD^2=r^2-(R^2/4)$ and finally

$$\theta_1 = \sin^{-1}\frac{\sqrt{r^2-R^2/4}}{\rho} = \cos^{-1}\frac{\sqrt{\rho^2-r^2+R^2/4}}{\rho} = \cos^{-1}\frac{q}{\rho}\text{; where}$$

$$q = \sqrt{t^2 - R^2/4}, \text{ and } t^2 = \rho^2 - r^2. \qquad N^1$$

θ_2 is found from triangle STO, which gives

$$ST = SO\cos\theta_2; \quad \theta_2 = \cos^{-1}\frac{ST}{SO} = \cos^{-1}\frac{t}{\rho}. \qquad O^1$$

The integral dose in zone 1 is

$$Di_1^s = A \int_0^{\cos^{-1} q/\rho} \frac{\sin\varphi}{2} \int_0^R e^{-\mu y}\, dy d\varphi = A \frac{\mu}{2\rho} R(\rho - q)\left[1 - \mu\frac{R}{2} + \mu^2 \frac{R^2}{6}\right]. \qquad P^1$$

The integral dose in zone 2 is found by first determining the integration limits along a ray, i.e. the length of MN. Dropping a normal to this chord from the centre of the sphere gives us two triangles.
Triangle MFO gives

$$MF^2 = r^2 - OF^2;\ MF = \sqrt{r^2 - \rho^2 \sin^2\varphi} = \sqrt{\rho^2\cos^2\varphi - \rho^2 + r^2}$$
$$= \sqrt{\rho^2\cos^2\varphi - t^2}, \qquad Q^1$$

where $t^2 = \rho^2 - r^2$.

$$Di_{11}^s = A \int_{\cos^{-1} q/\rho}^{\cos^{-1} t/\rho} \frac{\sin\varphi}{2} \int_0^{2\sqrt{\rho^2\cos^2\varphi - t^2}} e^{-\mu y} dy d\varphi =$$
$$= A\frac{1}{2\rho}\left\{\frac{Rq}{2} - t^2 ln\frac{q + R/2}{t} - \mu\left[\frac{2q^3}{3} + \frac{4t^3}{3} - 2t^2 q\right] + \right.$$
$$\left. + \mu^2\left[\frac{R^3 q}{24} - \frac{Rt^2 q}{4} + \frac{t^4}{4} ln\frac{q + R/2}{t}\right]\right\} \qquad R^1$$

Summation for the two zones gives the total dose as

$$Di_{S.A}^s = A\frac{1}{2\rho}\left\{R\rho - \frac{Rq}{2} - t^2 ln\frac{q + R/2}{t} - \mu\left[\frac{R^2(\rho - q)}{2} + \frac{2q^3}{3} - 2t^2 q + \frac{4t^3}{3}\right] + \right.$$
$$\left. + \mu^2\left[\frac{R^3\rho}{6} - \frac{R^3 q}{8} + \frac{t^4}{2} ln\frac{q + R/2}{t}\right]\right\}. \qquad (13)$$

Our formulae represent a first attempt to calculate β-ray doses exactly, allowing for absorption inside and outside the medium. The compilation of convenient numerical tables for the more commonly used β-emitters forms the subject of further work.

All the calculations are for point sources, and are therefore not applicable to beams such as are produced by accelerators. The same method can, however, be applied to parallel beams. Results for a sphere, cylinder and truncated cone have been derived, and will form the subject of a separate paper.

Translated by J. E. S. BRADLEY

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A UNIVERSAL ELECTROPHYSIOLOGICAL INSTRUMENT*

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THERE is an acute shortage of equipment capable of recording the slowly varying potentials produced by nerves in response to electrical stimulation. The oscillograph sweep has to be synchronized with the stimulating pulse; moreover, many such processes are best photographed from a single sweep, whereas a repetitive sweep is best for visual study. It must therefore be possible to switch over rapidly from single-sweep to repetitive-sweep operation without altering the sweep rate. Double pulses of adjustable relative delay are also required with many nervous processes. The designs and circuits that have so far been described [1–6] are often restricted to use for particular purposes, or are in many ways inconvenient. We therefore designed a universal apparatus based on the normal EO-7 cathode-ray oscillograph which is suitable for all normal purposes.

With this instrument one can (*a*) record single nerve potentials in single- or repetitive-sweep operation, with the sweep synchronized to the stimulator pulse, (*b*) stimulate with single or double rectangular pulses, with the pulses independently adjustable for height and duration, and with the delay variable, (*c*) use either X- or γ-plates, so a miniature camera (the FED, the Kiev, the Exacta, etc.) can be used for recording 6–10 oscillograms per frame, and (*d*) record the shapes of the stimulator pulses.

As scarce components are not used and the design is simple, the apparatus can be made in any physiology laboratory. The circuit (Fig. 1) consists of a thyratron relaxation oscillator (V_4, V_5), a horizontal sweep amplifier (V_{11}–V_{13}), a delay stage for the double pulse generator (V_6), the second pulse generator (V_9) and the output cathode follower stage (V_{10}). It is supplied from the a.c. mains; the stabilized H.T. is supplied by two valve rectifiers.

The circuit operates as follows. The thyratron oscillator (V_5) oscillates continuously when the gating valve V_4 is switched off, and the repetitive scan operates.

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Single-sweep operation occurs when V_4 is switched on; the thyratron is then fired by applying a positive voltage to its grid from the divider R_{18}, R_{19}, R_{22}, C_{10}. The voltage is applied by pressing a button; the sawtooth voltage appearing at the anode is fed to the X-amplifier (V_{11}–V_{13}). The pulse from the cathode of V_5 simultaneously triggers the double-pulse delay stage (multivibrator V_6). The delay is controlled by the chain C_{12}, R_{34}, R_{23}. When the multivibrator has operated, the second stage (V_7, first pulse

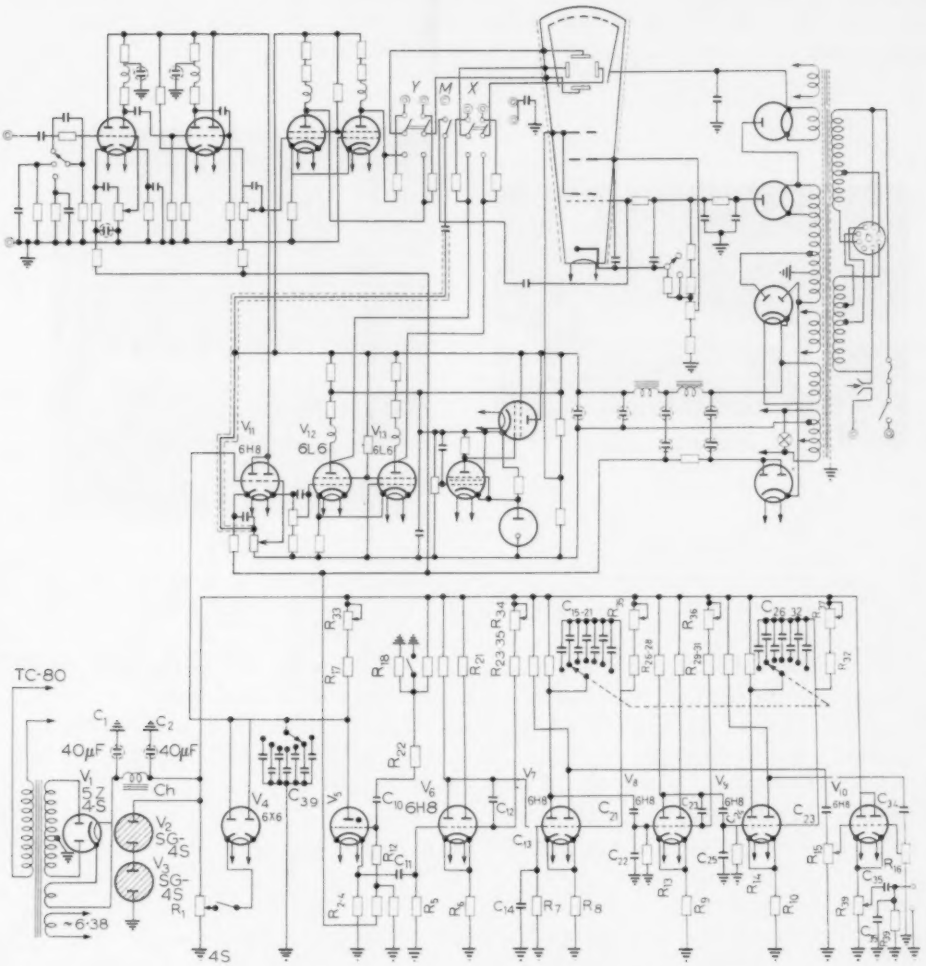

FIG. 1. The universal electrophysiological apparatus.

V_1, 5TS4C; V_{2-3}, SG4–5; V_4, 6X6; V_5, TG 0·1/0·3; V_{6-10}, 6H8; C_{1-2}, 40 μF, 450 V; C_{10}, 9100 pF; C_{11}, 1000 pF; C_{12}, 0·1 μF; $C_{13,\ 21,\ 24}$, 1200 pF.
R_1, 100 kΩ (var.); R_2, 10Ω; R_3, 900 kΩ; R_4, 43 kΩ; R_5, 3·6 kΩ; $R_{6,\ 8,\ 10}$, 2 kΩ; $R_{7,\ 13,\ 14}$, 6·2 kΩ; $R_{15,\ 16}$, 1 MΩ (var.); R_{17}, 1 MΩ; R_{18}, 51 kΩ; $R_{19,\ 22}$, 100 kΩ; R_{20}, 30 kΩ; R_{21}, 24 kΩ; R_{23}, 0·75 MΩ; $R_{24,\ 28,\ 31,\ 32}$, 24 kΩ; R_{33-37}, 4·7 MΩ (var.); R_{38}, 1 kΩ; R_{39}, 22 kΩ (var.); ch = choke.

generator) takes over. The voltage from the right anode is fed directly to the grid of the output cathode follower V_{10}; the duration of the first pulse is controlled by C_{15}–C_{21} and R_{35}, and the amplitude by the coarse and fine controls R_{11} and R_{15} respectively. The first pulse generator simultaneously operates the delay circuit to the second pulse generator (V_8). The delay can be varied over a wide range by C_{23}, R_{36} and R_{39}. The second pulse generator (V_9) then operates; C_{26}–C_{32} and R_{37} control the pulse duration, and R_{11} (coarse) and R_{16} (fine) the amplitude. The pulse is simultaneously fed to the second grid of the cathode follower. Fig. 2 shows the shapes of the first and second pulses, recorded by passing the pulses direct to the Y-amplifier.

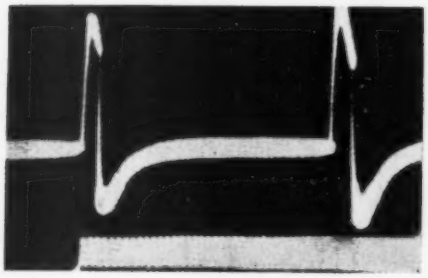

FIG. 2. Shapes of the double pulses (1000 c/s time marker at bottom).

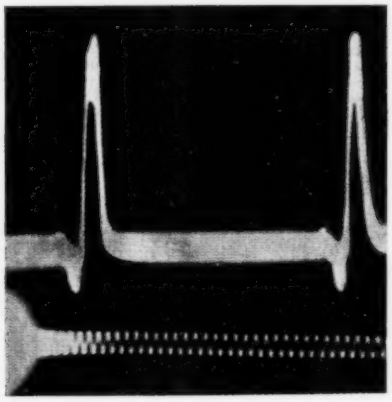

FIG. 3. Action potentials from the small tibial nerve in the cat on stimulation with double pulses (1000 c/s time marker at the bottom).

The horizontal shift is effected by using the variable resistor in the grid circuit of V_4 in the paraphase output amplifier, and the vertical shift by slightly unbalancing the output stage of the vertical amplifier. This latter amplifier was a U-2-00 low-frequency encephalograph amplifier (from the Experimental Workshop of the Academy of Medical Sciences of the U.S.S.R.); the anodes of the output stage were connected directly to the Y-plates.

Any similar amplifier could be used for the purpose. The cameras that can be used to record the oscillograms are the Kiev, the FED, etc. Fig. 3 shows two action potentials recorded from the small tibial nerve in the cat using a single sweep. The time-marker is a 1000 c/s signal applied directly to the Y-plates.

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LETTERS TO THE EDITOR

CHANGES IN THE MITOTIC ACTIVITY OF THE ROOT MERISTEM OF WHEAT SEEDLINGS AFTER IRRADIATION WITH X-RAYS*

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IT has been shown in work by Shekhtman and co-workers [1] that during irradiation of wheat seedlings with fast electrons, changes in the intensity of the radiation by $1{\cdot}3 \times 10^4$ times do not affect the radiobiological effect if the exposure is not for longer than 20 min. On the other hand, in investigations by the same author [2] it was established that the prolonged irradiation of the same wheat seedlings with X-rays for 4 hr or more reduces the radiobiological effect by 2–2·5 times compared with less prolonged exposures. Hence we consider that the rate of restoration in the wheat seedlings is very low and that the so-called "characteristic time" [3], i.e. the time during which the effect of action of the radiation falls by e times on account of reparative processes, is approximately 4–6 hr. Investigations carried out by means of ^{32}P [4] show that after irradiation with a dose of 500–1000 r, the permeability of the roots of the wheat is sharply diminished, but after 4–6 hr recovery begins and continues for 24 hr to almost the normal state.

From this point of view we were interested to investigate mitotic activity in the roots of wheat seedlings at different times *after* irradiation with X-rays.

Irradiation of 48 hr wheat seedlings *Erythrospermum* 599 was performed. Samples were taken before irradiation and then later, at intervals of 15 min, and 1, 5, 24 and 48 hr after irradiation. The material was fixed in a Navashin fixer. The number of dividing cells in a longitudinal central section of the root was determined. In each experiment 7–10 sections were counted. Altogether three series of experiments were performed, using pulsed and continuous irradiation. Pulsed radiation was given by

* Biofizika **3:** No. 4, 519–521, 1958.

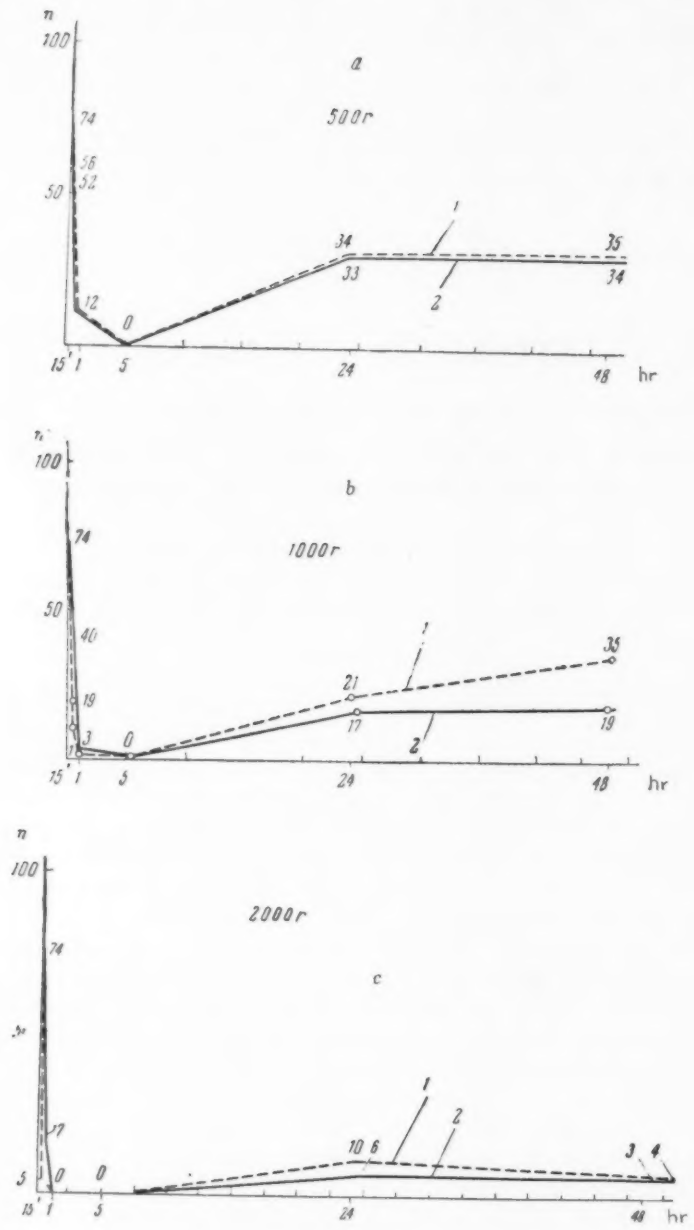

FIG. 1. Action of X-rays on the number of mitoses in the meristem of wheat roots. 1—pulsed radiation; 2—rectified half-wave radiation. *T*—time after irradiation (in hr). The figures on the curves are absolute counts of mitoses.

a method described earlier [1], with an apparatus with condensers. By means of a mechanical contact they were discharged through the X-ray tube, which was of the hot cathode T-200 type. Duration of the pulse was 0·1 sec and the pulse dose was 2·4 r. Continuous radiation was given from a rectified half-wave apparatus of the same average dose rate (about 40 r/min). The doses used were 500, 1000 and 2000 r.

The results of the observations are shown in Fig. 1, from which it can be seen that with all the forms of irradiation maximum suppression of the number of mitoses and the onset of recovery of mitotic activity appear 5 hr after irradiation. At a dose of 2000 r suppression of mitosis is practically irreversible.

Curves of mitotic activity of similar character after irradiation were found in a culture of animal tissues, as reported [5].

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SUMMARY

(1) During the action of radiation on mitotic activity in 48 hr wheat seedlings three phases of reaction were observed: (*a*) a rapid fall in the number of divisions in the first hour after irradiation; (*b*) a phase of absence of division; (*c*) a phase of recovery.

(2) Maximum suppression of the number of mitoses and the onset of recovery of mitosis appeared 5 hr after irradiation, which indicated a low rate of recovery of these processes in the roots of wheat seedlings.

(3) These findings are in agreement with the results of the investigations by Shekhtman [1, 2] in which it was shown that the "time factor" during irradiation of wheat seedlings appears only in cases where the duration of irradiation is not less than 4–6 hr.

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AUTOFLUCTUATIONS IN THE PROCESS OF PHOTOSYNTHESIS*

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IT is a noteworthy fact that photosynthesis, like other processes in plants, may in constant environmental conditions proceed at a variable, periodically fluctuating rate

* Biofizika **3:** No. 4, 521–523, 1958.

[1–3]. It is of interest to discover whether this fact could be the result of the autofluctuating character of the photosynthetic reaction itself or whether it is connected with overheating of the leaf, the activity of the pore apparatus and so on. In this short note we describe an attempt at theoretical examination of the problem based on the cycle of chemical conversions suggested by Calvin [4, 5].

It is well known that an essential condition for the appearance of autofluctuation is the autocatalytic character of the reaction. In the process of photosynthesis an autocatalytic action may take place. Calvin's cycle closes, i.e. the CO_2 acceptor regenerates itself during the process. It follows from this that photosynthesis is an autocatalytic reaction, i.e. any increase in the concentration of any of the intermediate products, in particular triose, pentose, etc., brings about an increase in the rate of its accumulation, and a fall in the concentration—a fall in the rate.*

We undertook the attempt to elucidate this process, taking as our basis Calvin's scheme and supplementing it with a number of hypotheses: connecting it firstly with respiration and, secondly, with the process of transfer of energy from chlorophyll to glycerophosphoric acid. It must be pointed out that the solution is purely illustrative in character. For each compound entering into the cycle, the equation of the rate of change of concentration was described. In this way the order of the reaction (i.e. the character of the relation of rate to concentration) was chosen from stereochemical considerations. Next, using the assumption that the products of the cycle with the longest life are triose-phosphate and hexose-phosphate, we expressed the concentration of the remaining compounds through the concentration of these main products. The assumption was also made that at certain points the process is reversible. As a result two equations for the concentration of triose-phosphate (c_3) and of hexose-phosphate (c_6) were obtained:

$$\frac{dc_3}{dt} = \alpha_1 c_3^2 - \alpha_2 c_3 c_6 + \alpha_3 \tag{1}$$

$$\frac{dc_6}{dt} = \beta_1 c_3^2 - \beta_2 c_6^2 - \beta_3 c_3 c_6 \tag{2}$$

where $\alpha_1 c_3^2$ is the variation between the two processes (the first being the formation of triose from ribulose and CO_2 with the participation of a nucleoprotein as a hydrogen donor,† and the second being a decrease in the triose on account of the formation of fructose-diphosphate); α_1 is a constant coefficient depending on the intensity of light and the concentration of CO_2 in the air; $\alpha_2 c_3 c_6$ is the decrease in triose-phosphate on account of its reaction with hexose-phosphate, leading to the formation of ribulose and tetrose, according to Calvin's cycle; α_3 is the constant influx of triose on account of hydrolysis of polysaccharides during respiration; $\beta_1 c_3$ is the conversion of hexose into disaccharide or, further still, into starch; $\beta_2 c_3 c_6$ is the loss of hexose in accordance with Calvin's cycle.

* Evidence that photosynthesis is an autocatalytic process appeared in the literature as long ago as 1952 [6].

† Strictly speaking the rate of this process is proportional to $c_5 x$ (where x is the concentration of the nucleoprotein transferring hydrogen to the c_5-ribulose). However, thanks to the closure of the cycle the concentration of ribulose c_5 itself is proportional to the concentration of triose. Regarding the nucleoprotein we suggest that it is formed from triose or ribulose and so its concentration also is proportional to c_3. It is impossible to discuss this in greater detail, since the chemical mechanism of protein formation during photosynthesis is not yet known.

In an equilibrium condition the rates $\frac{dc_3}{dt}$ and $\frac{dc_6}{dt}$ will be zero, i.e. just as much of the compound is added in a unit of time as is lost. All the concentrations are conveniently measured as fractions of the equilibrium concentrations, i.e. by considering that under stationary conditions c_3 and c_6 are equal to unity. The conditions that these rates in an equilibrium process are zero place the following restrictions on the coefficients α and β:

$$\alpha_1-\alpha_2+\alpha_3=0$$
$$\beta_1-\beta_2+\beta_3=0.$$

Further, from stereochemical relationships a connexion may be found between the values of β_2 and β_3. Actually in an equilibrium process, with each circuit of Calvin's cycle one molecule of CO_2 is taken up. Consequently, for the conversion of one molecule of hexose into starch, six complete cycles are required. In each cycle

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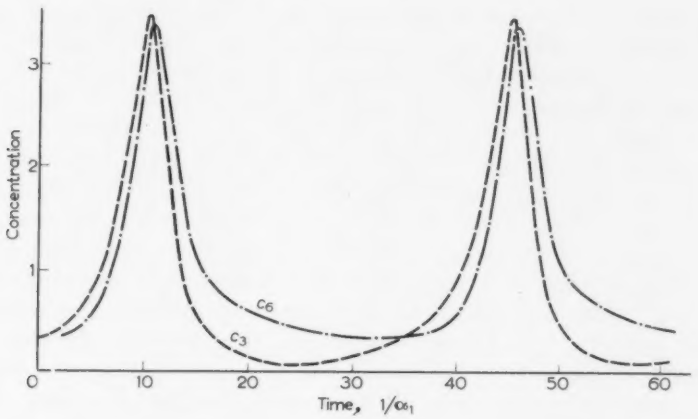

FIG. 1. Relation of the concentration of triose-phosphate (c_3) and hexose-phosphate (c_6) to time. Ordinate: concentrations c_3 and c_6 in fractions of the equilibrium values. Abscissa: time in units $1/\alpha_1$ ($1/\alpha_1$, by this system of values, is equal to the time of half circuit of Calvin's cycle).

the quantity of the compound converted into starch must be six times less than the quantity involved in the cycle. Thus:

$$\beta_2=\frac{1}{6}\beta_3;\ \beta_2=\frac{1}{7}\beta_1;\ \beta_3=\frac{6}{7}\beta_1.$$

So far as the coefficients α are concerned, it can be stated that α_3 is bound to be less than $\alpha_1/7$, since the quantity of the compound entering the cycle on account of hydrolysis of polysaccharides must be far less than the quantity of polysaccharides formed during the process of photosynthesis. Otherwise the productivity of photosynthesis would be negative. We assigned the value $\alpha_3=\alpha_1/20$. The value of α_2 was subsequently determined in the same way and was equal to 21/20 of α_1.

Using the numerical values of these coefficients we investigated the resistance of the stationary condition of the process by the method of small fluctuations. It was

found that this condition is stable if α_1 is less than $1{\cdot}2\,\beta_1$, and unstable otherwise. The coefficient α_1, as it appeared, is proportional to the intensity of light. Hence it follows that with increased illumination photosynthesis will be transformed from a stable stationary process into an unstable, autofluctuating system with periodic depressions. We investigated an unstable system, assigning the value $\alpha_1 = 1{\cdot}3\,\beta_1$. The system of equations was solved graphically by the method of isoclines.

A stable cycle was produced, and on this basis the relation of the concentrations of c_3 and c_6 to time was considered. This relationship bears a well-marked periodic character. The rate of photosynthesis, with the given assumptions, is proportional to the concentration of c_3. The general form of the relationship resembles the mid-day depression of photosynthesis due to intensive illumination (Fig. 1).

The calculation which we have made is illustrative in character, since it contains a number of assumptions. It must be emphasized which of these assumptions are essential and which are not. The assumption that the concentration of nucleoprotein (hydrogen donor) is proportional to the concentration of triose is very essential. This is based on the hypothesis that protein (especially the nucleoprotein hydrogen donor) is itself constantly formed during photosynthesis. Otherwise the first term of equation (1) would be proportional to the first degree of c_3 and the process in this case would be stable and autofluctuations would never be observed. The assumption of a connexion with respiration (the term α_3) is also essential, since this term α_3 plays the part of an expendible catalyst. In its absence photosynthesis could not begin. Its absolute value is not so important. It may also be pointed out that with a diminution in α_3 the period of depression is lengthened, and with its increase it is shortened, i.e. this means that photosynthesis can only emerge from depression if there is a certain quantity of triose or carbohydrate which is convertible into triose in the leaf. There is some evidence in the literature [7, 8] on the necessity for introducing an "expendible catalyst". It is known, for example, that in a high intensity of light, or during transfer of plants from the dark, a high intensity of photosynthesis does not develop at once; it grows gradually. It follows from this that an "empty" leaf assimilates less intensively than one which has accumulated a certain quantity of the products of assimilation. The remaining assumptions do not play an essential role.

SUMMARY

(1) The hypothesis of the autofluctuating character of the process of photosynthesis gives an explanation of the rhythmic nature of the process which is observed experimentally, particularly the appearance of mid-day depression, which is attributable neither to the pore mechanism nor to overheating of the leaf, etc.

(2) The hypothesis enables a number of conclusions to be drawn from a study of the periodicity of photosynthesis under constant conditions, concerning the kinetics of the chemical reactions, and in particular, concerning the actual order of any of the reactions in the kinetic sense (in other words, how the rate of the process depends on the concentration of its products).

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THE RADIOBIOLOGICAL EFFECT OF INTERRUPTED IRRADIATION BY X-RAYS*

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STARTING with the investigations of Talbot (1834), interrupted irradiation has been widely used in physiology, in particular in the study of the mechanism of photosynthesis [1, 2]. In radiobiology in recent years several papers [3–5] have been devoted to the investigation of the effect of interrupted irradiation. These have shown that during interrupted irradiation there takes place a well-marked frequency effect, characterized by the fact that at a definite frequency of interruption of the radiation and at a definite relation between the "light" and "dark" intervals, the radiobiological reaction is at a maximum or minimum.

In accordance with the cumulative theory of a "time factor", developed in the various works [6–8], the radiobiological effect must depend on the average dose-rate, and the effect of the frequency of interruption, if it does occur, must be uniform in character, without any well-marked maxima or minima.

In order to test this hypothesis we undertook the investigation of the influence of interrupted irradiation on the growth of the roots and shoots of wheat.

EXPERIMENTAL METHOD AND RESULTS

For the experiments we used 48 hr wheat shoots of the *Erythrospermum* 599 variety. The seed was sown and cultivated after irradiation by a method previously described [7, 9]. As an indicator of the radiobiological reaction, root growth was used. Measurements were made of the length of the longest root 7 days after soaking the seed.

Irradiation was carried out by an X-ray tube, using hard rays with a half-value layer of 1 mm Al (wavelength = 0·58 Å). The maximum dose rate was 170 r/min in air at the point where the object was placed. Irradiation was for 6–24 min

* Biofizika **3:** No. 4, 524–526, 1958.

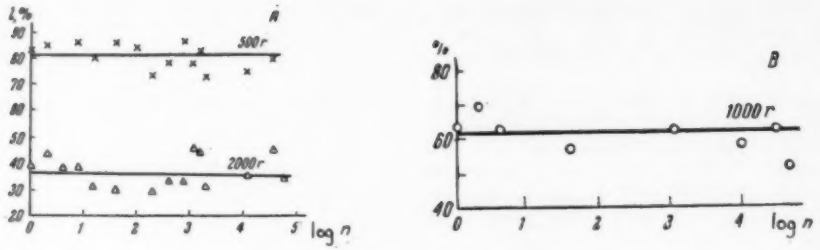

FIG. 1. Relationship between the biological effect and the frequency (n) of an interrupted beam of X-rays. Ordinate: length of the roots of wheat shoots (l, in per cent of control).

(depending on the dose selected). The total duration of irradiation and the average dose-rate with uninterrupted and interrupted irradiation were the same, and were regulated by the focal distance of the object. Both forms of irradiation were produced by the same apparatus.

In order to obtain interrupted irradiation a brass disc with cut-out sectors was used. The thickness of the discs was from 1 to 5 mm. Preliminary investigations showed that with the least thickness of disc of 1 mm, not more than 4 per cent of the total quantity of radiation passed through the opaque part, which was taken into consideration in the form of a correction at the time of irradiation. The frequency of interruption was varied by the number of sectors and revolutions of the disc, and could be altered from 1 to 60,000 interruptions per min. The width of the sector corresponded to the ratio between the "light" and "dark" interval (1: 1). Thus the duration of the "light" interval was changeable from 30 sec to 5×10^{-4} sec. The rate of revolution of the electric motor was controlled by a tachometer.

During irradiation the shoots were placed in a ring-shaped gutter of a dish made of organic glass, 20 mm in width and with a radius of 60 mm, which permitted equal irradiation of all the shoots. During irradiation the shoots were protected from drying by means of moist filter paper.

The results of the experiments using interrupted irradiation are shown in Fig. 1. As can be seen from the figure, change in the frequency of interruption did not have any noticeable effect on the radiobiological reaction of the shoots.

Together with the investigation of the effect of interrupted irradiation,

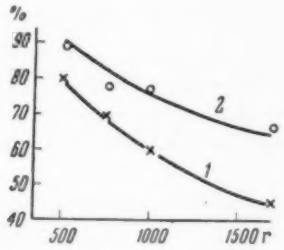

FIG. 2. Action of brief (1) and prolonged (2) irradiation with X-rays in various doses on the biological effect. Ordinate: length of roots of wheat shoots (l, in per cent of control).

observations were made to compare brief (6–24 min) and prolonged (4–7 hr) irradiation on the same object.

The results of the experiments on prolonged irradiation are shown in Fig. 2, from which it can be seen that for a certain dose, lengthening of the time of irradiation to 7 hr gives a well-marked influence of the "time factor", as follows: the ratio of the length of the roots in brief and prolonged irradiation at a dose of 1000 r is equal to 0·78, i.e. brief irradiation acts 22 per cent more effectively than the prolonged. The Schwarzschild coefficient, calculated for a 37 per cent dose, is equal to 0·873, which agrees with the results of previous investigations [7]. Brief irradiation was performed before and after prolonged irradiation, and the average results obtained. The root length during the second brief irradiation (after the prolonged) was slightly greater than during the first, which indicated some degree of change in the radiosensitivity of the shoots during the 7 hr, i.e. during the irradiation. However this difference was insignificant.

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DISCUSSION

As can be seen from the experiments on prolonged irradiation, deviations from the law of mutual replaceability take place only during prolonged periods of irradiation of 4–7 hr. If these deviations are connected with the presence of reparative processes in the cells and tissues of the shoots, it is clear that these processes take place very slowly, at least in that part of the destructive processes which are reversible in character. These results are in agreement with those of other workers [9–12].

Change in the frequency of interruption from 1 to 60,000 per min and in the duration of the "light" and "dark" interval from 30 sec to 5×10^{-4} sec did not, therefore, have any influence on the radiobiological effect, as was actually shown in our investigation. However, since in a number of reports [3–5] a well-marked frequency effect was observed, it may be supposed that this discrepancy was connected with the special features of the radiobiological reaction and metabolism of different organisms, and in particular with the possibility of the occurrence of certain chain reactions during irradiation whose intermediate products have a mean duration of life of the order of $1–10^{-3}$ sec, which is in agreement with the experimental findings of Sutton [13] and Ghormley [14]. So far as the findings [15] of Denier* are concerned, they are obviously the result of errors of method, possibly dosimetric in nature.

In conclusion I express my gratitude to Prof. Ya. L. Shekhtman for his guidance and help in the work, and the discussion of the results.

SUMMARY

(1) During the action of interrupted irradiation with X-rays on wheat shoots, changes in the frequency of interruption from 1 to 60,000 per min, with a ratio of 1 : 1 between the "light" and "dark" intervals (the duration of the interval being from 30 sec to 0·5 msec) did not appreciably affect the inhibition of root growth.

(2) In comparing brief and prolonged irradiation, the "time factor" begins to influence the effect when the time of irradiation is increased to 4–7 hr, which is in agreement with the results of investigation of the permeability of wheat roots [10] and the mitotic activity [11] after irradiation.

Translated by B. HAIGH

* Denier found a twofold increase in the radiobiological effect on plant shoots of interrupted irradiation of low frequency, as compared with uninterrupted irradiation.

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DISCUSSION

CONCERNING THE LETTER BY I. G. KRASNYKH AND S. P. IARMONENKO PUBLISHED IN BIOFIZIKA (VOL. 2, NO. 6, p. 764, 1957)*†

IN Biofizika No. 6 of 1957 there was published a critical letter by I. G. KRASNYKH and S. P. IARMONENKO referring to our article "Reduced sensitivity to lethal doses of X-rays in animals previously given sublethal doses" (Biofizika **1:** No. 3, 1956).

This criticism is not concerned with the substance of the work, since, as Krasnykh and Iarmonenko write, they accept the possibility which is pointed out by them in the paper reviewed of increasing the resistance of animals to the action of radiation by preliminary irradiation. Our findings were obtained in 1950–1951, but unfortunately were published only in 1956. In the interim a number of papers confirming our conclusion appeared in the literature (M. Betz, 1950; E. Cronkite, *et al.*, 1950; P. N. Kiselev, P. A. Buzini and K. I. Nikitina, 1956; L. A. Dimitrov, 1957; and so on).

All the critical observations of the reviewers relate to the frequently occurring problem of the doses and conditions of irradiation used, which are not specially dealt with in the paper. It must be acknowledged that it would be useful to tackle the problem of unification of conditions of irradiation, although to obtain full agreement on this problem would evidently not be easy. It is well known that the biological effect from the same dose depends not only on the conditions of irradiation but also on a number of other factors (initial physiological condition of the animal, seasonal

* Biophysics **2:** 733, 1957.

† Biofizika **3:** No. 4, 527–528, 1958.

variations, special features of the animal, position of the source, diet and so on). As regards this, the number and arrangement of the sources of radiation are very important (uniformity of irradiation of the object) and also, within definite limits, as shown in particular by our work (Dokl. Akad. Nauk SSSR **103:** No. 3, 1955), the dose-rate. It must be pointed out that variations in the dose-rate, taking place in the work under review (10–25 r/min), could not in any way affect the results obtained, as the reviewers indicate.

The authors of the letter should be aware of the extremely contradictory Soviet and foreign literature on the dose of radiation which is lethal for different species of animal. Thus for rats, according to the recommendation of the Ministry of Health of the U.S.S.R., the LD 50/30 is 640–450 r. Meanwhile, in work by Ross, Aikel *et al.* (1953) rats irradiated with 1600 r survived for more than 12 days. According to Mole and Batt (1953) a dose of 600 r kills only 10 per cent of rats irradiated. Andriiasheva (1957) and Fokina (1957) observed rats irradiated with a dose of 500 r for a period of 23–30 days and even found restoration of the initial level of haemoglobin and erythrocytes, and these investigations were continued for a longer period. After the same dose the absence of a lethal outcome in rats and mice was also observed by G. V. Voskoboinikov (1955), V. N. Sivertseva (1956), D. M. Merimova (1956), Mytareva (1956) and so on.

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N. E. Ponomarenko (1956), N. N. Blokhin and co-workers (1956), and also Syromyatnikova (1957), irradiating dogs with a dose of 500 r, did not observe death of the animals, although the normal course of metabolism was upset. Consequently, the absence of death of animals even with a dose of 500 r has been observed not only by us but by a number of workers.

Unfortunately, in the letter by Krasnykh and Iarmonenko unscrupulous citation of our articles can be found. Thus, to quote one of their citations: "[Animals] given 500 r remained active, ate their food well and did not lose weight." But the following phrase is omitted: "although this amount of radiation did not cause death of the animals it led to substantial changes in metabolism" (p. 235). The reviewers also did not mention that in Table 2 results are given of the number of animals in each variant of the experiment.

Krasnykh and Iarmonenko accuse us of not pointing out the precise conditions of irradiation (in fact they are given on p. 233), but in speaking of their own findings the authors of this letter mention absolutely no conditions under which they carried out irradiation of dogs. It is also worth mentioning that the reviewers did not use doses recommended by the Ministry of Health of the U.S.S.R. (1954). According to this recommendation, the LD 50/30 for dogs is 300–400 r. The authors of this letter irradiated 13 dogs with a dose of 500 r (higher than the dose causing 50 per cent mortality) and only 5 dogs died, i.e. 38 per cent.

The anxiety of Krasnykh and Iarmonenko that our findings may mislead a wide circle of readers can hardly be treated seriously, in view of the extremely contradictory nature of the data in the literature on the lethal dose. Every specialist, working in this field, must above all begin an experiment to establish the relationship between the reaction being studied in the animals used, and the dose of irradiation and the experimental conditions, as was done in the case of our own investigation. The second irradiation of the animals was carried out in our experiments with an

absolute lethal dose, giving 100 per cent death of control dogs and rats, and the number of these controls used was perfectly adequate (see Tables 1 and 2).

We earnestly request the Editorial Committee to insert this reply in the pages of Biofizika to dispel the misunderstandings which might arise from reading the letter from our critics.

B. M. GRAEVSKAIA and R. IA. KEILINA

Translated by B. HAIGH

THE ROLE OF WATER IN THE STRUCTURE OF COLLAGEN*

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THE collagen-group proteins show a highly specific chain configuration; we have shown that this configuration only occurs in isolated areas where imino acid and glycine residues accumulate [1, 2]. Grassman's recent outstanding work [3] has also demonstrated that such accumulations occur.

Detailed study of the chain configuration shows that some problems related to hydration remain to be solved, particularly since air-dried collagen contains much bound water (some 25 g per 100 g protein), which can form hydrogen bonds to polar groups in the protein and thus link the structure together. Certain data also indicate that the structure is very sensitive to the water content; thus, Katz long ago stated that the 2·9 Å reflections vanish when the cold form of gelatin is vacuum dried [4]. The evidence [5] that completely dry collagen preparations have largely disordered structures points to the same conclusion. On the other hand the specific configuration can be restored to initially amorphous hot gelatin, but only after a certain amount of water has been added [6]. These data do not derive from special studies of the effect of water, however, although they indicate that water plays a special part in collagen and related proteins. The extensive X-ray data indicate only that the early stages of hydration see an increase in the distance between chains and a loss of orientation relative to the texture axis [5, 7, 8].

The structure studies must therefore include a careful examination of the role of water in the areas with the specific configuration; this forms the subject of the present paper.

The main method used was to measure the absolute intensity changes in reflections corresponding to the specific structure which accompany water content changes. The redistribution occurring in the relative intensities was studied simultaneously. Sorption isotherms and curves of uptake kinetics were also taken, to give data as complete as possible. The X-ray work was done in the Department of Solid State Physics of the Physics Faculty at Moscow State University; the sorption work was done in the Colloid Chemistry Laboratory at the Karpov Institute of Physical Chemistry. Some preliminary results have already been published [2].

EXPERIMENTAL

(1) *X-ray studies.* Collagen and procollagen were used. The collagen was rat-tail tendon (RTT) fibre, washed in distilled water. Orientated specimens were

* Biofizika **3**: No. 5, 529-540, 1958.

prepared by drying under tension; unorientated ones were prepared by carefully chopping up the fibres. The procollagen was produced in the Protein Biochemistry Laboratory at the Institute of Biochemistry, Academy of Medical Sciences of the U.S.S.R., by the method of Orekhovich *et al.* [9]. Films of salt-free protein 10–15 μ thick were prepared from a solution in 0·01 N acetic acid made up from the material resulting from washing and centrifuging the precipitate obtained by dialysis against tap water several times. A previously cleansed mercury surface was used for the purpose. Pure CuK_α radiation was used, as the soft Cu component is more strongly absorbed in dry material than is the residual hard "white" component remaining in ordinary filtered radiation, which results in a strong diffuse ring from the hard components. The monochromatic radiation was produced by reflecting the primary beam from a flat pentaerythritol crystal placed in a specially designed holder directly in front of the X-ray tube window [10]. The photographs were taken in an RKSO chamber, rigidly fixed in position for all specimens.

The apparatus of Fig. 1 was used to ensure a defined relative humidity. The specimens were located in perspex boxes, with two slots cut in them, one vertical, one horizontal. The vertical one held a solution with a known saturation vapour pressure, while the horizontal one allowed the X-ray beam to pass; the specimen was placed at the end of this. The first slot was wide enough for the effects of surface tension on vapour pressure to be negligible. The vertical holes were seated with vacuum plasticine, and one of the horizontal ones with a monocrystal mica sheet not more than 15 μ thick. The other was closed with a 25 μ Al foil, which also served as an intensity standard. The solution was introduced via a hole in the top of the box using a syringe. The P_2O_5 (when used) was first placed in a special container and inserted in the slot. Two specimens (unorientated RTT and unorientated procollagen) were examined simultaneously in two cameras. In each case a series of photographs was taken at various humidities; adequate time was allowed for equilibration before the photographs were taken. The time was known roughly from the sorption kinetics measurements; the time required for the structural changes to go to completion was determined from the photographs. Flat cassettes with a specimen to film distance of 40 mm were used. The X-ray tube was operated at 40 kV, 12 mA, and gave exposures of 3–6 hr (lesser values at the higher humidities). The intensities were measured over the range $(\sin\theta)/\lambda = 0{\cdot}02$–$0{\cdot}3$.

The texture of the Al foil was not removed even by very careful annealing, so a steel wire (Fig. 1) was fitted to the cassette, and the intensity of the standard measured in areas near where the shadow of the wire intersected the Debye rings. Calibrated absorbers were placed in the central area, to bring the intensities onto the straight part of the film response. An MF-2 microphotometer was used to measure all intensities.

The beam was not of true circular section, although circular stops were used, because the primary beam was reflected from a flat crystal; consequently, the intensities were somewhat non-uniformly distributed round the rings. All films were therefore photometered along six radii, and comparisons made along each such direction; the results were then averaged. The experimental intensities were corrected; since Lorenz factors calculated as for a regular crystalline solid would not be strictly correct, we evaluated the intensities as functions of θ, and applied appropirate

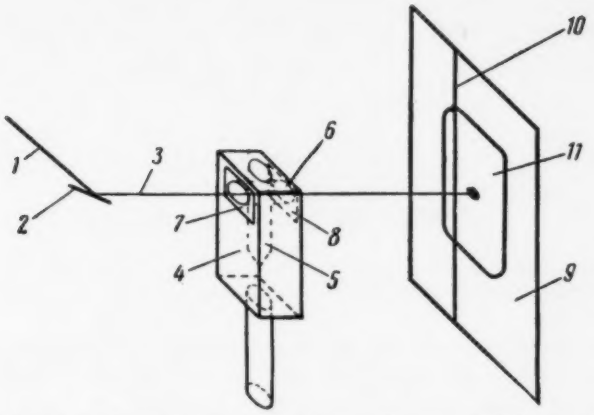

FIG. 1. Method used for photographing moist specimens. (1) Primary white X-ray beam, (2) crystal monochromator, (3) monochromatic beam, (4) perspex cell, (5) vertical channel in cell containing solution of fixed vapour pressure, (6) specimen, (7) single-crystal mica, (8) Al foil, (9) cassettes, (10) steel wire, (11) calibrated absorber.

corrections, instead of finding the integral intensities. These intensities as functions of θ were determined using constant slit widths on the monochromator; for flat films the relationship is then, $I \propto \text{cosec}\ \theta \cot \theta$, where θ is the angle of reflection.

Intensity curves were thus drawn up for three specimens of unorientated RTT collagen, and for procollagen, at different humidities. A set of results for orientated RTT collagen was also obtained. The results for the first two materials can be summarized as follows.

(1) Collagen and procollagen show almost identical types of variation with humidity, but the times required for the two materials to reach steady states differ; procollagen requires 24 hr, whereas collagen needs 2 weeks. The intensity changes found with procollagen are very much sharper than those for collagen (Figs. 2 and 3).

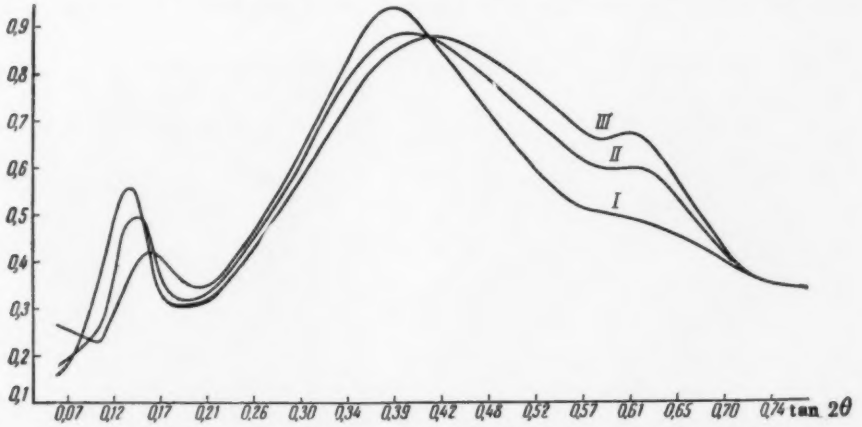

FIG. 2. Intensities of maxima of disorientated RTT collagen at three humidity values. (I) Specimens kept over P_2O_5, (II) air-dried specimens, (III) relative humidity 81 per cent. Abscissa: tan 2 θ.

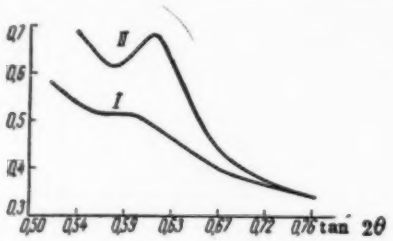

FIG. 3. Variation in intensity of the $d = 2{\cdot}9$ Å maxima with humidity for procollagen specimens. (I) Air-dried specimens, (II) relative humidity 81 per cent. Abscissa: tan 2 θ.

(2) As the humidity increases, (a) the reflections corresponding to the specific configuration (i.e. the main equatorial and 2·9 Å reflections) increase in absolute intensity, (b) the diffuse ring decreases in absolute intensity, and the general background weakens, (c) the 2·9 Å reflections increase in intensity relative to the main equatorial one and, (d) the 5·8 Å reflections decrease in intensity relative to the main equatorial one; this effect is unrelated to the changes in the diffuse ring, since the ratio of the two intensities alters.

The orientated collagen results can be summarized as follows.

(1) A diffuse untextured ring appears at $(\sin\theta)/\lambda \sim 0{\cdot}13$ when the collagen is dried over P_2O_5; the structure becomes more amorphous.

(2) The reflections corresponding to the ordered areas become much weaker; in unorientated specimens they tend to fuse into rings, particularly those for $d \sim 4$ Å. All the effects are completely reversible.

The increase in d with humidity found for the main equatorial reflection is familiar; some points are worthy of note. The reflections corresponding to $d \sim 11$ Å spread out along the equator as the humidity increases; the maximum shifts to larger d-values, and becomes flatter. The 5·8 Å reflections are difficult to detect at high humidities, because their intensities drop.

(2) *The adsorption isotherms.* The sorption and desorption isotherms were taken

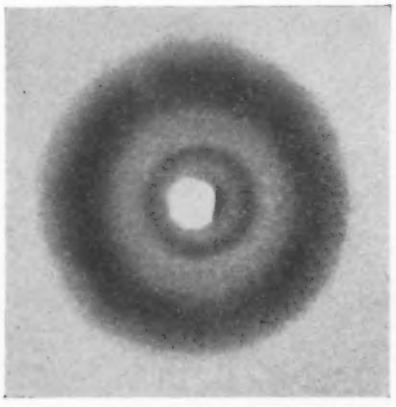

FIG. 4. RTT collagen stored over P_2O_5.

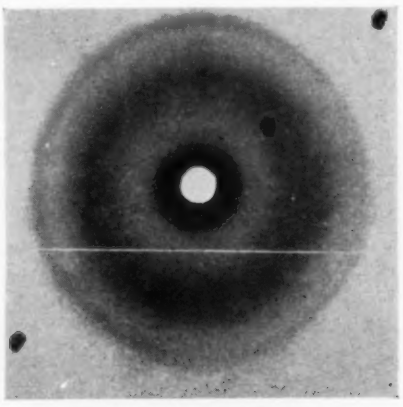

FIG. 6. Procollagen at 81 per cent relative humidity.

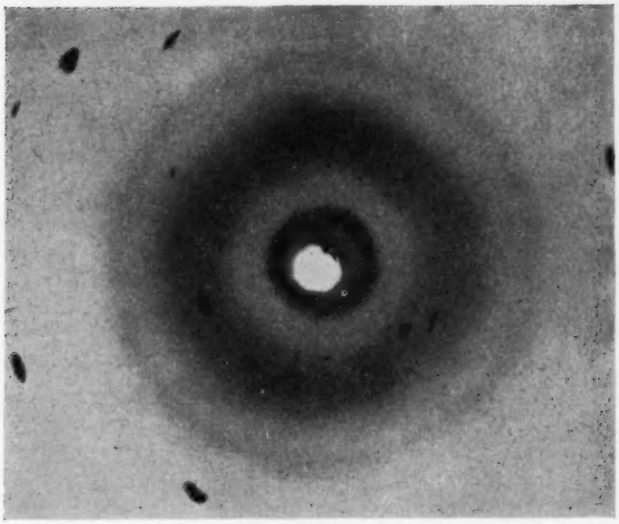

FIG. 5. Air-dried RTT collagen.

for the materials used (15–20 μ procollagen films and RTT collagen fibres first teased out as fibres 40–50 μ thick). The spring balance method [12] was used.

Kinetic curves were first taken for each point on the sorption and desorption curves; these measurements were also made for the residue from RTT collagen

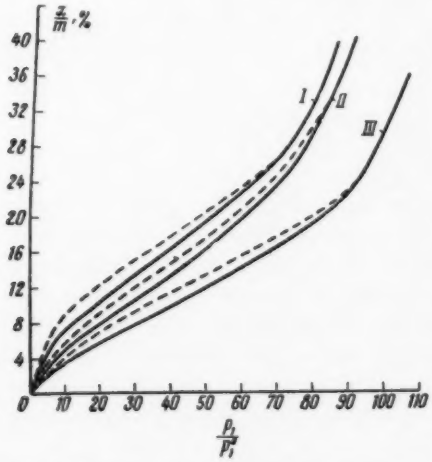

FIG. 7. Adsorption and desorption isotherms (latter dashed) for water and procollagen (I), collagen (II) and collagen residue (III). Abscissa: relative vapour pressure, $p_1/p_1°$. Ordinate: amount of water absorbed per 100 g of specimen.

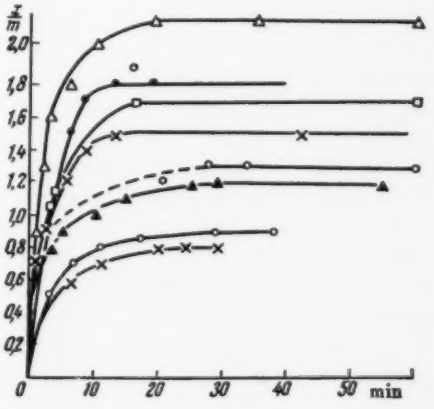

FIG. 8. Sorption kinetics of water on procollagen at various relative humidities. Ordinate: amount of water absorbed.

remaining after the soluble protein had been exhaustively extracted. The results were as follows.

(1) The isotherms were of the S-shape typical of many proteins and polymers (Fig. 7).

(2) Procollagen absorbed the most water, and the collagen residue the least, at any given water vapour pressure.

(3) The curves show a certain amount of hysteresis.

(4) The kinetic curves always show a distinct saturation region, reached at 20 min with procollagen, in 24 hr with collagen, and after 4–5 days with collagen residue (Figs. 8 and 9).

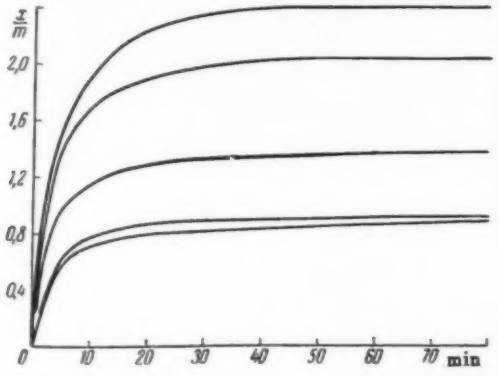

Fig. 9. Sorption kinetics of water on collagen at various relative humidities. Ordinate: amount of water absorbed.

DISCUSSION

Bonding of water molecules to polar groups is of primary importance in the sorption of water on proteins [7, 13–15]. The results are discussed in the light of the idea that the specific collagen configuration arises in isolated areas showing accumulations of amino-acid residues [1–3]. The intensities of reflections arising from such areas are regularly related to the humidity; the specific structure is lost or distorted as the water is removed, so the water must act to stabilize the special structure.

A comparison with poly-L-proline and with polypeptides containing glycine and L-proline [16] is of interest. Several configurations are found, including one with a large negative specific rotation (as with the specific configuration in collagen); this configuration occurs only in certain solvents, so the regular left-handed spiral must result from some specific interaction. The structures and solvation mechanisms of these polypeptides and of the special areas in collagen must therefore have much in common.

The way the intensities vary on hydration shows that the water molecules are not simply enclosed between the side-groups of adjacent chains, since the resulting water interlayers would scatter in the opposite phase to the chains and reduce the intensities of the odd orders, while intensifying the even orders. In fact, the first-order equatorial reflection is actually intensified, so the water must at least in part be placed not more

than one quarter-period away from the chain axes (i.e. about 3 Å). The first-order equatorial reflection might also be intensified because the main equatorial reflection is shifted to small angles, where the Fourier transform of the molecule is large. However, the meridional reflections do not shift during swelling, though their intensities relative to the main equatorial maximum increase. Some of the intensity change is due to additional scattering by the water; the water must be so placed that the second maximum on the equatorial line shows a loss in intensity for dry specimens. This also shows that the water must lie about 3 Å from the molecular axis, in the specific areas, at least. This is to be expected, since areas where the imino acids and glycine are concentrated do not contain many of the polar groups available. The hydroxyproline OH groups form hydrogen bonds to free CO groups [17]; the position of the bonds are such as to hinder the approach of water molecules.

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Some of the CO groups in the specific structure areas are free because the pyrrolidine N does not participate in hydrogen bonding, while the other CO–NH groups form structures which support this configuration, as infra-red data [18] show. The stability of the specific structure during hydration is largely determined by the strength of the CO–NH bond system. Some of the energy liberated by bonding water molecules must be used to weaken, or even break, the hydrogen bonds in solvation, whereas our data indicate that the structure becomes less deflective as hydration proceeds. We must therefore suppose that areas with the CO–NH bonds are not solvated; steric difficulties must therefore prevent the water getting in. The role of configurational difficulties in solvation has recently been well illustrated for the α-proteins [19].

Our sorption data indicate that the number of water molecules bound to areas with the specific configuration corresponds to the number of CO groups not bound to NH or hydroxyproline OH groups. The total number of residues in a RTT collagen chain of weight 10^5 is 1214 [20], and of these 667 lie in areas with the specific structure [15], while the other 547 are distributed over the rest of the molecule. The amorphous, open-structure areas are hydrated first; these contain many of the polar groups. Table 1 presents some solvation* calculations for these areas. The total number of water molecules absorbed by the disordered areas is 1482, i.e. equivalent to 26,670 water units per protein unit of 10^5. Now 100 g of dry protein absorbs 30 g of water at 81 per cent relative humidity; of which 26·6 g is present in disordered areas and only 3·4 g in areas with the specific structure. Hence the 667 residues in areas with the

TABLE 1. SOLVATION CALCULATIONS

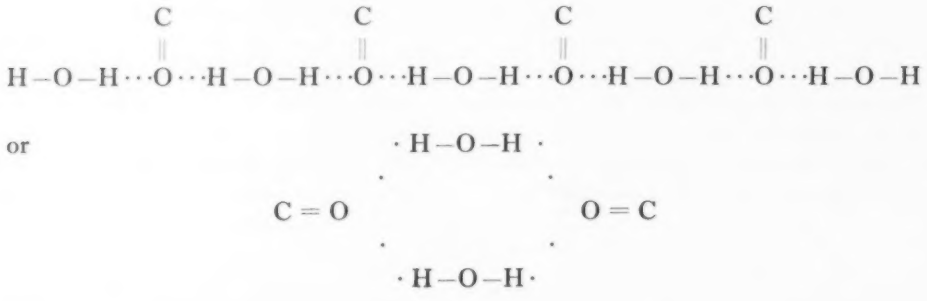
Polar group	No. of groups per area of weight ($\times 10^5$)	No. of water molecules per group	No. of water molecules absorbed
Peptide	547	1	547
OH	69	3	207
COOH	139	4	556
Basic group	86	2	172

* The ordered areas contain practically all the imino acid residues, and about an equal number of glycine residues [1–3].

specific structure (782 polar groups) take up 188 molecules of water; far from all the polar groups are solvated at 81 per cent relative humidity. The groups first to be solvated are naturally the free ones, or the most accessible ones; i.e. the CO groups not involved in hydrogen bonds, of which there are 151. If all CO–NH groups absorb water equally, we should require one molecule per group [7, 15]; however, the sorption capacities of the peptide links in ordered and disordered regions differ, and a peptide group can take up at least two molecules of water [15, 21]. If the peptide links in ordered and disordered regions differ, we would expect those in the disordered regions to take up extra water more rapidly above 80 per cent relative humidity, so that they would have two water molecules each, and not one. The others remain without water molecules. This latter deduction is confirmed by the fact that the specific configuration is retained, and with it the stabilizing hydrogen bonds, even in solution [22].

The ordered regions in collagen thus absorb relatively little water, and that in close proximity to the main chain; CO groups not involved in hydrogen bonds must be responsible*. As the specific configuration can only occur in limited areas of the molecules, the water must be unevenly absorbed along the chain. This is indicated by data on low-angle X-ray scattering, where the intensity distribution changes rapidly with humidity; this would not occur if the water was uniformly distributed. Bear [27] has repeatedly stated that the capacity to absorb water is unevenly distributed along collagen fibres.

Hydrogen bonds can be formed between the water molecules and the chain bonds as the water molecules lie so near the axis (about 3 Å away). Hydrogen bridges may then partially stabilize the structure [7, 28], and the water may play a part similar to that in crystalline hydrates [29]. The water molecules may be bound to CO groups as shown below



Oxalic and acetylenedicarboxylic acids show the second type of structure in their hydrates (for example, see [29]). But the first scheme is more probable on energy grounds, since the O atoms have larger effective negative charges than in the second. Most crystalline hydrates have continuous chains or closed rings of hydrogen bonds [29].

* The model proposed by Crick [23], Ramachandran [24] and Cowan [25] has two-thirds of the peptide groups not taken up with hydrogen bonds. Cowan *et al.* [26] have shown that all the hydrogen bonds can be formed by adding water molecules to the CO groups.

There is one molecule of water per CO group in the above schemes, but the water bound by the protein is inadequate to supply the CO–NH groups in the areas of specific structure. A collagen fragment of weight IO^5 takes up 1700 molecules of water at 81 per cent humidity, while if each CO group has one molecule of water, while the CO–NH groups in the areas of specific structure have none, the figure should be 2090. Bridges can occur between chains if the groups which take up the water are 5–6 Å apart. This imposes certain restrictions on the model.

We determined whether water so tightly bound could be removed simply by reducing the humidity as follows. Fine-grained Rochelle salt powder was placed over P_2O_5; this salt has a structure largely stabilized by hydrogen bridges [29]. After about a month the structure had broken down completely; this was determined by X-ray methods. The water bound to the protein is undoubtedly more easily removed than this.

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The water must be semiregularly placed along the chain because the 2·9 Å spots become stronger relative to the main equatorial ones; the sorbing groups must be about 3 Å apart along the chain axis. The chains move further apart on hydration, and our data indicate that any water taken up in the specific structure areas goes mainly onto the main chain, and not onto the side-chains. It may be that the sorption on side-chains in other areas (which are still orientated, of course) will tend to force the chains as a whole apart. This will increase the spacing in the ordered areas as well.

We finally consider the data from the adsorption isotherms obtained after exhaustively extracting the soluble protein; they indicate that, as the soluble material (procollagen) is removed, the sorption capacity falls, the procollagen films have structures more open than those of collagen fibres. The data as a whole show that procollagen and collagen behave, respectively, like pure materials and like ones incorporating impurities essential to the structure. The collagen residue after extraction must naturally contain the largest amount of such impurities, and the shapes of the sorption and kinetic curves agree with this, since procollagen reaches equilibrium in 20 min, whereas collagen requires 24 hr. The structural changes in the areas of specific configuration take a day to complete in procollagen, and two weeks in collagen. Taking these results with those of [30], we may say that some of the structural changes found in the collagen group proteins only occur slowly.

SUMMARY

(1) The absolute and relative intensity changes with humidity in the X-ray reflections from collagen have been determined, to establish the role and position of the water in the areas of specific structure.

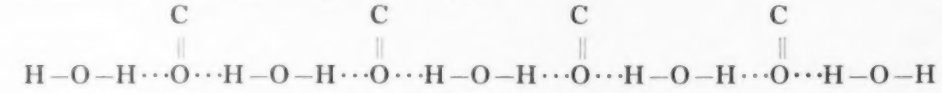
(2) Sorption isotherms and kinetic curves have been taken for collagen, procollagen and collagen exhausted of soluble protein.

(3) The spots corresponding to areas of ordered structure are found to increase in absolute intensity with increase of humidity, and to show a change in the distribution of their relative intensities.

(4) The results indicate that the water plays a large part in stabilizing the specific configuration by forming hydrogen bridges.

(5) The way the intensity distribution changes with the humidity indicates that the water in the areas of specific configuration lies very close to the chain (not more than 3 Å away); the water molecules are arranged in a semiregular fashion along the chain (some 3 Å apart).

(6) The water molecules are most probably bound to main-chain peptide groups. Calculations of the water absorbed, and comparison with experiment, indicate that only the CO groups not involved in hydrogen bonds in the areas of specific structure take up the water, at least at 81 per cent humidity. The ordered areas may then be stabilized by hydrogen bridges of the type



which gives rise to continuous chains of structurally sorbed water.

Translated by J. E. S. BRADLEY

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* There exists a translation into English of this work.

STRUCTURAL AND PHYSICO-CHEMICAL FEATURES OF PROTEINS OF THE ACTOMYOSIN COMPLEX*

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THE data accumulated in the last 15–20 years indicate that the contractile proteins in muscle show alterations in their molecular structures during contraction. Contraction has been mostly studied from the chemical aspect, and much work has been done since the discovery of the ATPase activity of myosin [1]. Much less work has been done on the structures of the contractile proteins. Although X-ray methods, electron microscopy and light scattering have been extensively used, the processes still remain obscure. Flow birefringence studies and methods of differentiating internal anisotropy from structural anisotropy while allowing for the effects of the solvent on the flow birefringence, have revealed some new features of the muscle proteins [2]. We give here some results from flow birefringence studies on the actomyosin complex.

METHODS

The myosin was prepared from rabbit muscle by Liubimova's 10 min extraction method; the purity and uniformity of the protein were checked by ultracentrifugation. The actin was prepared by Straube's method; F-actin polymerized in 0·1 M KCl was used to determine the flow birefringence. Artificial actomyosin prepared by combining pure myosin and actin was used. The flow birefringence was determined in an apparatus with an internal rotor; the optical system has already been described [2]. Low protein contents (0·2 to 0·01 per cent) could be used, because the sensitivity was high. The solutions were diluted with either 0·1 M or 0·5 M KCl to give an approach to zero velocity and concentration conditions ($n/(gc\eta_0)$ for $g \to 0$, $c \to 0$). The viscosities were determined in an Ostwald viscometer. The refractive indices were adjusted by adding aniline to glycerol solutions; the maximum value which could be produced while keeping the solution transparent was 1·56, i.e. almost that for dry myosin (for which Weber gives 1·57). This was done to differentiate internal anisotropy from structural anisotropy.

EXPERIMENTAL RESULTS AND DISCUSSION

The myosin molecule is highly asymmetric, as the flow birefringence, the high viscosity, and the light scattering indicate. Flow birefringence studies give the orientation α of the optical axis of the particle to the optical axis of the solution and to the flow direction, and the flow birefringence proper, Δn; both these are functions of the velocity gradient, of the concentration and of the nature of the solvent.

* Biofizika **3**: No. 5, 541–546, 1958.

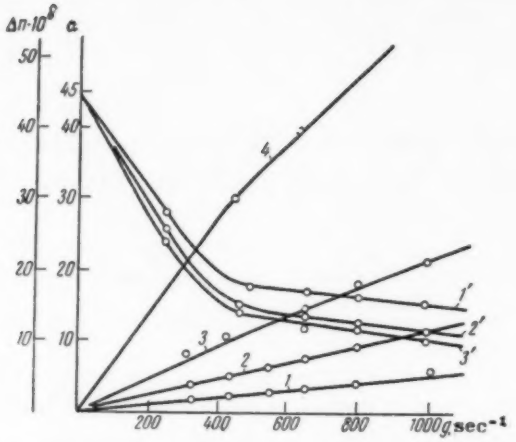

FIG. 1. Relation of magnitude and orientation of birefringence to velocity gradient for myosin in 0·5 M KCl. (1) 0·025 per cent; (2) 0·05 per cent; (3) 0·1 per cent; (4) 0·3 per cent.

Figure 1 shows the results we obtained; myosin gives a straight line in all cases. The concentration curve is strictly linear, which must mean that the myosin particles do not dissociate on dilution (Fig. 2).

The concentration-independence and anisotropy is specified by the constant

$$[n] = \lim (\Delta n/gc\eta_0)\ g \to 0,\ c \to 0$$

where g is the velocity gradient, c the concentration, and η_0 the relative viscosity of the solvent.

Myosin has a value of $2{\cdot}55 \times 10^9$ for this constant, which results from the summation of the internal and structural anisotropies of the myosin particles. The two contributions were differentiated by using solutions with different refractive indices consisting of glycerol, aniline and 0·5 M KCl.

Internal anisotropy in artificial myosin fibres and in glycerol-extracted single myofibrils has been detected using solutions of different refractive indices [3, 4]. Lauffer [5] has found that there is no flow birefringence at $n = 1{\cdot}55$ with tobacco mosaic virus. The effect has not previously been studied on pure muscle proteins; Fig. 3 shows our data on the way the flow birefringence varies with the refractive index of the solvent.

The internal component is $[n]_e = 1{\cdot}02 \times 10^9$, and comprises 40 per cent of the total. Myosin is inactivated by dilution with aniline, while tobacco mosaic virus

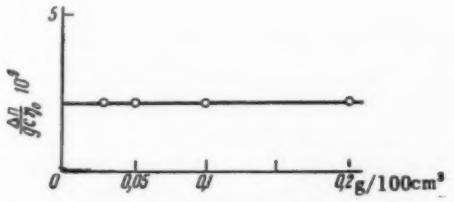

FIG. 2. Concentration dependence of the flow birefringence for myosin in 0·5 M KCl.

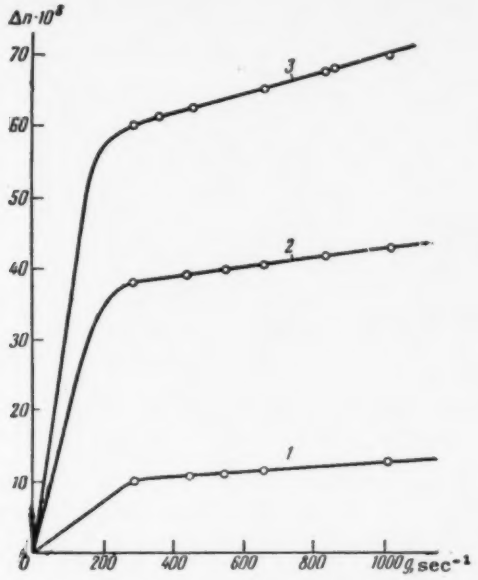

FIG. 3. Relation of birefringence to velocity gradient for F-actin. (1) 0·062 per cent; (2) 0·125 per cent; (3) 0·025 per cent.

remains biologically active, so our values for myosin can therefore only relate to the inactive state. Previous studies [6, 7] (as will be seen later) have mainly been done on actomyosin of variable composition, and not on pure myosin; some workers [8, 9] consider that the flow birefringence found with this myosin is therefore a consequence of the traces of residual actin, and that pure myosin should show no flow birefringence. We have shown that pure myosin shows a marked flow birefringence and internal anisotropy (in agreement with Joly *et al.* [10]); we have also found that L-meromyosin shows flow birefringence, so myosin must possess a characteristic ordered structure.

Actin. One of the most interesting features of actin is that it exists in two forms; globular (G-actin) and fibrillar (F-actin). Minute traces of salts transform the G-form to the F-form; linear aggregates of variable length result. Very high flow birefringence appears on diluting the G-form with 0·1 M KCl. Actin differs from myosin in the way the flow birefringence varies with g and c. Myosin gives linear relationships, whereas actin gives a saturation (constant) orientation even at quite low gradients, which is typical of molecules with high axial ratios, i.e. with very long particles. The F-form shows a tendency for the flow birefringence to fall with the concentration (Fig. 4), perhaps because the F-form may dissociate on dilution (Fig. 5). The effect is still more marked if distilled water is used instead of 0·1 M KCl; the value for the G-form is almost reached. The filaments of the F-form produced by polymerization are weak because the birefringence (which does not vanish instantly when the apparatus is stopped) tends to drop if high velocity gradients are applied for long periods; this effect is not found with myosin and actomyosin. The shape of the

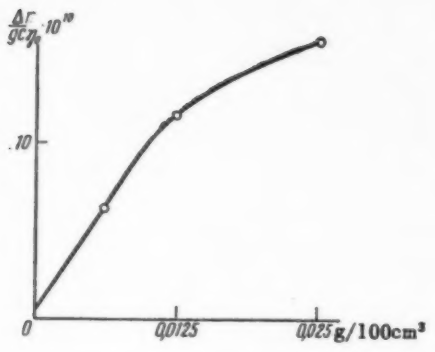

FIG. 4. Concentration dependence of the flow birefringence for F-actin.

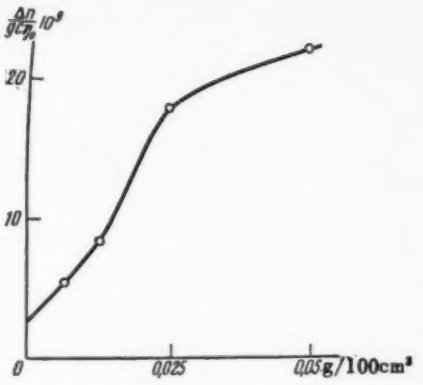

FIG. 5. Concentration dependence of the flow birefringence for actomyosin.

particles is solely responsible, as the refractive indices of solvent and solute differ; the effect disappears if the two are the same, so the molecule has no internal anisotropy. Figure 3 shows how the flow birefringence for the F-form varies with the refractive index of the solvent.

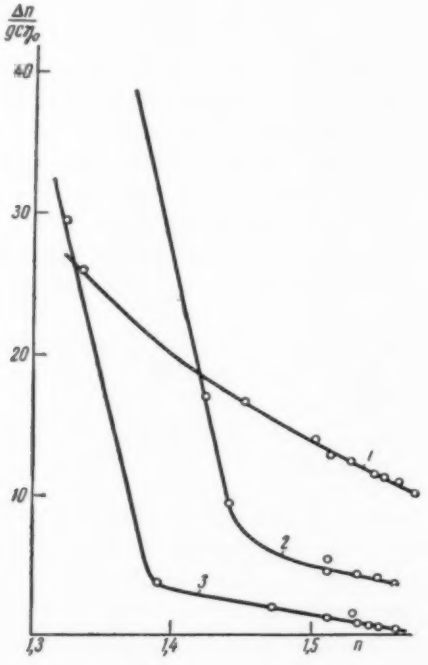

FIG. 6. Variation of $\Delta n/gc\eta_0$ with refractive index of solvent (1) for myosin, $\times 10^8$, (2) for actomyosin, $\times 10^9$, (3) for actin, $\times 10^{10}$.

Actomyosin. Myosin can combine with actin in varying ratios (different workers give optimum values varying from 1 : 3 to 1 : 6 [11]); the properties are thereby changed, particularly the viscosity, which rises anomalously and exceeds the sum of the viscosities of the components.

The complex was prepared by mixing myosin and polyactin in the ratio 1 : 4. This gave a fairly high concentration of myosin while retaining optimal complexing conditions; the effects from myosin, actin and actomyosin could thus be compared simultaneously. The resulting viscosity change is accompanied by a change in flow birefringence; the value lies between those for actin and myosin. Figure 6 shows how the flow birefringence of actomyosin varies with g; a saturation orientation is soon reached although the effect is not so marked as with the F-actin. The flow birefringence falls considerably on dilution, as does the viscosity; the use of solvents with different refractive indices indicates that for actomyosin $[n]_e = 3{\cdot}5 \times 10^9$, i.e. is more than twice as great as pure myosin. As the second component (actin) has no internal anisotropy, we must suppose that complex shows intramolecular changes as well as micellar association; it is known [12] that the appearance in the electron microscope changes when the complex is formed. The long, thin rods of F-actin are replaced by shorter branched particles of actomyosin; Tsao and Baily [13] suppose that the actin dimers split up into monomers at this stage. The lesser flow birefringence of actomyosin (relative to F-actin) is very probably related to rearrangement of the linearly associated particles, which results in the axial ratio altering considerably.

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SUMMARY

(1) Pure myosin shows a marked and stable flow birefringence; the particles have an internal anisotropy responsible for 40 per cent of the total anisotropy.

(2) G-actin shows traces of flow birefringence; F-actin shows rapid and complete orientation at low velocity gradients. The entire effect with actin is due to form alone; the particles have no internal anisotropy.

(3) Actomyosin shows a flow birefringence falling between F-actin and myosin. The actomyosin particles are internally anisotropic (more than three times as anisotropic as those of myosin), so some intramolecular rearrangement must occur.

Translated by J. E S. Bradley

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* There exists a translation into English of this work.

STUDY OF THE FORMATION OF FREE RADICALS BY PHOTOREDUCING CHLOROPHYLL AND ITS ANALOGUES USING CHAIN POLYMERIZATION INITIATION*

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THE reversible photoreduction of chlorophyll and its analogues, which results from electron transfer, and which gives a pair of radical ions was discovered in our laboratory [1]. Polymerization initiation and electron paramagnetic resonance (e.p.r.) methods have been combined with spectroscopic techniques in the study of the free radical intermediates. Uri has observed that chlorophyll can initiate the polymerization of methylmethacrylate, and that ascorbic acid assists it in doing this [2]. Oster [3] has found that monomers polymerize when illuminated in systems containing dyes and ascorbic acid. Dolgoplosk [4] has studied the use of dienols in redox systems for initiating polymerizations in the dark. Linschitz and Weissman [5] have studied the red photoreduced form of chlorophyll by e.p.r., while Commoner [6] and Calvin *et al.* [7] have used e.p.r. to study the free radicals formed in illuminated chloroplasts.

The present paper deals with the free radicals formed by chlorophyll which will initiate the polymerization of methylmethacrylate [8]. The very large " chemical amplification " obtained from this chain reaction is of value in detecting free radicals in systems to which spectral methods are inapplicable.

METHODS

Pigment preparation. Chlorophyll-*a* and chlorophyll-*b* were prepared from dry nettle leaves by our modification of Zscheile's [9] method, bacteriochlorophyll from a *Rhodopseudomonas* culture [10], protochlorophyll from the inner coats of *Curcubita* seeds [11], phycoerythrin from *Callithamnion* [12], and pheophytin by Fischer's [13] method. The pigments were separated and purified by chromatography on sucrose columns. Cytochrome-*c* was prepared from ox-heart muscle by the Keilin–Hartree method [14], which gives it in the oxidised state; ascorbic acid was used as reductant (using the increase in absorption at 550 mμ as control method) in phosphate buffer at pH 8·6 and 50 °C. Excess ascorbic acid was avoided by only reducing about 90 per cent of the cytochrome.

Methylmethacrylate purification. The stabilizer (hydroquinone) was removed by repeated washing with 10% soda solution until the washings were quite colourless (about 20 times the volume of monomer was usually required), followed

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by water washing and drying over $CaCl_2$. The monomer was then further purified by ultra-violet irradiation (using a PRK-2 lamp), to dispose of impurities at the beginning of photopolymerization, and distilled *in vacuo*. The ultra-violet treatment did not usually influence the results, however.

Solution experiments. In a Thunberg tube 2 ml of an ethereal solution of the pigment of appropriate concentration (usually 10^{-5} M) was evaporated *in vacuo*; 5 mg of the hydrogen donor and 2 ml of a methylmethacrylate–pyridine mixture (3 : 1) were then added. The solution was usually deaerated by vigorous shaking while under vacuum, and then illuminated with red light, using an RG-2 filter, at 10^5 erg/cm^2 sec, using a 500 W projector lamp plus condenser. The specimen was kept cool in a vessel containing circulating water at 20°C. Then 0·5 ml of the solution was poured into 5 ml of methanol, and the precipitated polymer was estimated by nephelometry. All possible control experiments were done, in order to get reliable and clear-cut results. In all cases, the controls done in darkness gave negative results, while those in the light containing chlorophyll and monomer only gave a slight effect, much less, however, than found in the full system.

Emulsion experiments. Water-soluble reductants were used in emulsion systems, with the chlorophyll dissolved in drops of the monomer. The excessive cloudiness occurring with ordinary emulsions was avoided by making the aqueous phase a 46% solution of sucrose, or a 50% $MgCl_2$ solution; these solutions have the same refractive index as methylmethacrylate. The emulsifier was a 0·5 per cent solution of egg albumin in M/15 Na_2HPO_4, or a 0·25% solution of serum albumin.

Firstly, 2 ml of an ethereal solution of the pigment of appropriate concentration (usually 10^{-5} M) was evaporated *in vacuo*; then 2 ml of freshly prepared methylmethacrylate was added. Following this 2 g of sucrose and 5 mg of hydrogen donor were added to 2 ml of protein solution; the product was added to the 2 ml of monomer and emulsified by vigorous shaking; water was added cautiously till the emulsion was quite transparent. The transparent green emulsion showed the characteristic red fluorescence of chlorophyll; it was deaerated at −10°C for 3 min (with vigorous agitation) and illuminated with red light as above for 15 min. If the polymerization was active the emulsion set to a gel; if it was less active 1 ml of glacial acetic acid was added and the product centrifuged at 3000 g for 10 min, to separate the phases. The polymer in the top (methylmethacrylate) layer was determined as above.

PHOTOSENSITIZED POLYMERIZATION

Chlorophyll–electron–donor system. Compounds soluble in pyridine–methylmethacrylate mixtures were used; ascorbic and dihydroxymaleic acids give the most vigorous reactions. Succinic, malic, oxalic, acetic, citric, glutamic and pyrotartaric acids, and sucrose, glucose, pyrocatechol and hydroquinone gave (*in vacuo*) effects similar to those for chlorophyll + monomer alone. Phenylhydrazine gave no polymerization, although photoreduction occurred.

Some biologically important, water-soluble donors could be used in the emulsions; dienols, cysteine and Fe^2 salts (oxalate, sulphate, Mohr's salt—probably all present as basic salts in phosphate buffer) were found to be highly effective. Control tests with the Fe^3 salts, or with the anions, gave negative results. The results with

Fe(II) salts were the most interesting; the elementary process supposed to occur is $\cdot X^{\cdot} + Fe^{2} \rightleftarrows X^{-} + Fe^{3}$, which probably cannot be detected spectroscopically because the back reaction is so fast. The chlorophyll-sensitized oxidation reduction of cytochrome [15] probably involves this process.

Proteins can also act as hydrogen donors; these are best used in emulsions, and, in fact, emulsions prepared with egg or serum albumin alone (without reductants) give a distinct effect. Cytochrome is very much more active. Here the reaction may be with the SH groups in the protein.

All the above experiments were done on air-free materials; of systems containing air, only solutions containing ascorbic acid showed polymerization. No emulsions polymerized.

Chlorophyll–electron–acceptor system. Nitro-compounds and Fe^{3} salts were used as oxidants, to see if chlorophyll could be reversibly photo-oxidized to give free radicals. Emulsions and solutions were used as above. In these experiments 2 ml of solution of 4 ml or emulsion (10^{-5} M in chlorophyll) containing 5 mg of oxidant was illuminated for 30 min via a KS-11 filter. The oxidants giving not much more effect than the blank were potassium ferricyanide, KNO_3, ferric sulphate, $MnSO_4$, ferric ammonium alum, ferric phosphate and oxalate, while all nitro-compounds, such as *o*-nitrophenol, dinitronaphthalene, 2:4-dinitrophenylhydrazine and *m*-dinitrobenzene inhibited the polymerization completely.

Electron–donor–chlorophyll–electron acceptor system. Electron (hydrogen) transfers from donor to acceptor via reversible reduction of chlorophyll have been studied in our laboratory [16]. The system most used has ascorbic acid as electron donor and riboflavin, saffranin or pyridine dinucleotide as acceptor; these latter compounds react rapidly with reduced chlorophyll to give more stable compounds—hence the dark reactions are slow. It might be expected that saffranin or riboflavin would inhibit polymerization in the absence of air because the free radical photoreduced forms would rapidly react with the acceptor; this is actually so (Table 1). To check that these substances did not in fact terminate the chains instead of reacting as supposed, we tried saffranin–ascorbic–acid–monomer and riboflavin–ascorbic–acid–monomer

TABLE 1. EFFECTS OF SAFFRANIN AND RIBOFLAVIN ON THE *in vacuo* POLYMERIZATION OF METHYLMETHACRYLATE

Pigment	Reductant	Acceptor	Polymerization at 20°C		K*
			Light	Dark	
Chlorophyll	Ascorbic acid	Riboflavin	—	—	1·0 (670 mμ)
Chlorophyll	Ascorbic acid	Saffranin	—	—	—
Chlorophyll	—	Riboflavin	++	—	—
Chlorophyll	—	Saffranin	++	—	—
Chlorophyll	Ascorbic acid	—	+++	—	—
Saffranin	Ascorbic acid	—	++	—	10·0 (540 mμ)
Riboflavin	Ascorbic acid	—	++	—	10·0 (448 mμ)

* K = extinction coefficient for a 1 cm path at the wavelength given.

Notes. Illumination time 30 min.

+++ indicates a gel with emulsions or gelatinous mass with solutions, ++ a homogeneous cloudy suspension, assayed by nephelometry, + traces of polymer.

systems, irradiated at the absorption maxima of the coloured acceptors, as these can also be photoreduced. Here the acceptors actually initiated the polymerization; the compounds also failed to inhibit thermal polymerization at 40°C.

These compounds might be expected to be ineffective in aerated materials, since oxygen acts as the final acceptor; this is actually so. The initiators here must be reduction products derived from oxygen, e.g. HO_2 or OH. The inhibition mechanism in anaerobic conditions is thus that the compounds react rapidly with the photoreduced chlorophyll.

Comparison of pigments. Protochlorophyll gives the most active polymerization under these conditions (Table 2); bacteriochlorophyll acts like chlorophyll, while pheophytin is less active. The results are related to the reactivities of the reduced forms; pheophytin gives a more stable reduced form than does chlorophyll [17].

Water-soluble haematopophyrin was used only in emulsions, with the pigments in the aqueous phase; it initiated polymerization well, so the radicals produced in the aqueous phase initiate the process at the interfaces with the droplets.

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TABLE 2. COMPARISON OF PIGMENTS

Pigment	Electron donor	Filter	Medium	Polymerization	
				Light	Dark
Protochlorophyll	Ascorbic acid	OG = 4	Solution	++++	—
Protochlorophyll			Solution	++++	—
			Emulsion	+++	—
Chlorophylls *a* and *b*	Ascorbic acid	RG = 2	Solution	+++	—
Chlorophylls *a* and *b*			Emulsion	+++	—
			Solution	+	—
Bacteriochlorophyll	Ascorbic acid	RG = 5	Solution	+++	—
Bacteriochlorophyll			Emulsion	+++	—
			Solution	+	—
Pheophytins *a* and *b*	Ascorbic acid	RG = 2	Solution	++	—
Pheophytins *a* and *b*			Emulsion	++	—
			Solution	—	—
Phycoerythrin	Ascorbic acid	ZS = 2	Emulsion	—	—
Phycoerythrin + chlorophyll	Ascorbic acid	ZS = 2	Emulsion	—	—
Haematoporphyrin	Ascorbic acid	CZS = 2	Emulsion	+++	—

Notes. The tests were done *in vacuo* with the pigment at 10^{-5} M and 5 mg of ascorbic acid; solutions illuminated for 30 min, emulsions for 15 min.

The phycoerythrin acted as emulsifier as well (only emulsions were used); small amounts of albumin were without effect. The pigment never gave any effect; this is related to its inability to be photoreduced, which was demonstrated in our laboratory [12].

Lifetime and nature of the initiators. Here the monomer was introduced into the mixed pigment and reductant after the illumination (at room temperature), or else the mixture of all three was illuminated at −40°C (i.e. in conditions where the primary electron transfer can occur [18], but where the polymerization cannot proceed), and the process then allowed to go in the dark at 20°C. Only pheophytin showed an effect which occurs because the reduced form is very long-lived; the photochemical

TABLE 3. INITIATION PRODUCED BY PRODUCTS REMAINING AFTER ILLUMINATION AT 20°C *in vacuo*

Pigment	Illumination time before adding monomer (min)			
	1	5	10	30
Chlorophyll	—	—	—	—
Protochlorophyll	—	—	—	—
Bacteriochlorophyll	—	—	—	30 sec
Pheophytin	++	—	—	++

Note. The 10^{-5} M pigment solution in pyridine and the ascorbic acid were placed in the lower part of a Thunberg tube, with 3 ml of monomer in a side-arm. The monomer was mixed rapidly with the pyridine solution after illumination and the mixtures kept at 20°C for 30 min.

properties of pheophytin have been studied in detail by Evstigneev and Gavrilova [19]. Chlorophyll and protochlorophyll give very stable products, but these cannot initiate polymerization (because they are so stable); the secondary reduced forms are produced from the primary active ones by back-reaction the instant the light is cut off.

In the experiments at −40°C, the primary reduced form could only accumulate with pheophytin; the secondary reduced forms are produced from the primary active ones by back-reaction in the chlorophylls, so their absorption spectra cannot be detected. The results of Table 4 show that only pheophytin gives polymerization, so the relation of initiation to lifetime is clear. Since Evstigneev and Gavrilova's experiments show that the primary product from pheophytin reacts instantly with oxygen [19], the effects of admitting oxygen before and after adding the monomer were tested by admitting about 0·1 ml of air to the Thunberg tube. This gave 0·02 per cent O_2 in the gas phase over the solution. Air admitted before pouring in the monomer inhibited the reaction; if added afterwards it was without effect (Table 5). This again shows that initiation is caused by the primary form. Under other conditions (O_2 present) products from the photosensitized oxidation produce initiation.

The initiation mechanism. The chain initiation mechanism can be represented as $\dot{X}^- + M \rightarrow \bar{X}\dot{M} + H^+ \rightarrow HX\dot{M}$, where X is chlorophyll and M monomer. The combination of X radicals with the polymer has been detected spectrophotometrically, since chlorophyll (with its absorption maximum shifted from the 665 mμ found in

TABLE 4. INITIATION PRODUCED BY PRODUCTS REMAINING AFTER ILLUMINATION *in vacuo*

Pigment	Illumination time (min)				
	At −40°C				At 20°C
	0·5	1	5	10	1
Pheophytin	—	++	++	++	—
Chlorophyll	—	—	—	—	++
Protochlorophyll	—	—	—	—	++
Bacteriochlorophyll	—	—	—	—	++

Note. Pigment concentrations 10^{-5} M, 5 mg ascorbic acid, dark reaction time 30 min at 20°C.

acetone to 655 mμ) has been detected incorporated in the polymer. This chlorophyll could not be extracted by repeatedly dissolving the polymer in acetone and precipitating with methanol. However, thermal polymerization (in darkness), using monomer containing chlorophyll, gave the same result. It is therefore difficult to say whether the chlorophyll is incorporated on account of the primary photoreaction, or via some secondary reaction.

The above results indicate that the primary forms, of radical ion type [19], are responsible for initiation. Two methods can be applied to determine how methylmethacrylate reacts with such radicals, namely (1) kinetic studies on the photoreduction of chlorophyll, with or without monomer (a method used in our laboratory

TABLE 5. EFFECT OF OXYGEN ON THE ACTION OF THE PRIMARY FORM OF PHEOPHYTIN

Air admitted	Illumination time	Pheophytin
After adding monomer	30 sec	++
	5 min	+
Before adding monomer	30 sec	—
	5 min	—

Evstigneev's results show that the primary form is highly reactive in pheophytin [19, 20]; the experiments were done as before, using 0·5 ml of monomer poured into 4 ml of pheophytin (10^{-5} M) in pyridine after illumination for 30 sec at −40° C.

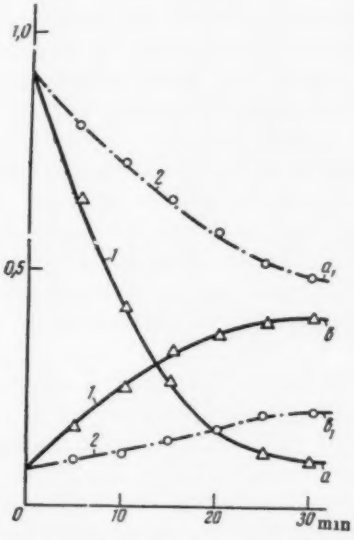

FIG. 1. Effect of methylmethacrylate on the photoreduction kinetics of chlorophyll. Curves (*a*) and (a_1) are the changes in *K* at 670 mμ (chlorophyll), (*b*) and (b_1) those at 525 mμ (photoreduced form of chlorophyll), (1) Test in pyridine, (2) plus 5 per cent methylmethacrylate.

for studying chlorophyll sensitization)—if acceptors are present the reduced form accumulates more slowly; (2) studies on the direct reaction between monomer and primary form (possible only with pheophytin). We have used both methods; in the first case we added 5 per cent of monomer to chlorophyll plus ascorbic acid in pyridine, and compared the kinetics with those found without the monomer. The light intensity was reduced to 10^3 ergs/cm^2 sec by inserting an NS-9 neutral filter, as the difference in the photoreduction cannot be detected at high light intensities. Figure 1 shows that the monomer quite clearly slowed down the fall in the red absorption maximum of the chlorophyll, and the rise in the 525 mμ maximum for the reduced form. The monomer therefore acts as electron acceptor as regards the reduced form. The value of K was measured at 470 mμ; the monomer was found to react with the primary form. When pheophytin + ascorbic acid + monomer (in pyridine) was illuminated at −40°C or at room temperature with 10 per cent D_2O added [20] (where the formation of the secondary form is retarded) no effect on the formation of the primary form could be detected; no initiation was found to occur in these conditions, either.

DISCUSSION

Polymerization is initiated by radical ions derived from the pigment reacting with the monomer in the chlorophyll–electron-donor–methylmethacrylate system. Other types of radicals may occur in this reaction, and their role in initiation must be considered. Examples are (1) a metastable biradical form of the pigment, formed, according to Terenin [21] and Lewis and Kasha [22], by $X + h\nu \rightarrow X^* \rightarrow {}^{\cdot}X^{\cdot}$; (2) a reductant radical ion (AH^+) is also formed by electron transfer: ${}^{\cdot}X^{\cdot} + AH \rightarrow \dot{X}^- + AH^+$; (3) peroxides or HO_2 and O Hradicals may be formed when oxygen is present. These possibilities will now be considered.

Biradical form of pigment. Methylmethacrylate containing only chlorophyll (lacking reductant and pyridine) polymerize on illumination, but the amount of polymer produced is very small. The biradical form is very likely responsible here [23], although another explanation is possible, namely that the monomer (or in general the solvent) acts as electron donor, since potentiometric measurements on pigments in pyridine show that negatively charged electrode-active particles are formed [24]. Oxidants also inhibit polymerization in monomer-pigment systems, perhaps because they react rapidly with the photoreduced forms. Rosenberg's [25] suggestion that the triplet state of the pigment may be able to initiate polymerization in systems containing ascorbic acid has not been confirmed by experiment; an effect is only found in systems containing an electron donor, and the solvent can act as donor in any case.

Reductant radical ion (AH^+). If reductant radicals were responsible for initiating polymerization, the nature of the pigment ought not to influence the polymerization rate, since the same initiator would be responsible in all cases; this is actually found to be so. It is hardly to be expected that the activity of AH^+ will be high, as Dolgoplosk [4] states that polymerization is only initiated by redox systems giving radical pairs if only one active radical is formed (otherwise rapid radical combination occurs). In this case the pigment gives the active radical (photoreduced form), and so the polymerization rate should be related to the properties of the pigment if a fixed hydrogen donor is used. It is thus unlikely that AH^+ initiates polymerization; this

would agree with the data on redox systems containing dienols which can initiate polymerization in the dark [4].

Part played by oxygen. The chlorophyll–ascorbic—acid–methylmethacrylate system polymerizes vigorously when air is present, so it can scarcely be doubted that radicals resulting from the reduction of oxygen participate. Polymerization occurs if the monomer is added after the system has been illuminated, when no photoreduced pigment can be present. Traces of oxygen are probably always present with the normal method of evacuating the Thunberg tubes.

A scheme that has been proposed for the photoreduction of oxygen in chlorophyll–dienol systems is [26]

$$\cdot X \cdot AH \rightleftarrows \dot{X}^- + \dot{A}H^+,$$
$$\dot{X}^- + O_2 \rightarrow \dot{X}O_2 \rightarrow X + \dot{O}_2,^-$$
$$\dot{O}^-_2 + H \rightarrow H\dot{O}_2 \rightarrow H_2O_2 + O_2, \text{ etc.}$$

If peroxides and OH or HO_2 formed under our (vacuum) conditions, the radicals could initiate polymerization; to check on this we carefully removed all oxygen from the system by illuminating the monomer in the side-arm with ultra-violet light for 10 min before transferring it into the chlorophyll + ascorbic acid in pyridine, cooled to −40°C. This procedure had no effect on the degree of polymerization. Since 0·02 per cent of oxygen inhibits polymerization when pheophytin–ascorbic acid is used in the same way, whereas there is no effect if the oxygen is added after the monomer, the active form must be the photoreduced pigment.*

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SUMMARY

(1) Free radicals are formed and initiate chain polymerization in methylmethacrylate when chlorophyll and its analogues are reversibly photoreduced.

(2) The primary photoreduced forms (radical ions) derived from the pigments initiate polymerization in oxygen-free systems, whereas peroxide radicals formed from the oxygen are responsible when oxygen is present.

(3) Ascorbic and dihydroxymaleic acids, cysteine and compounds of Fe^2 produce the most energetic polymerization in pigment–hydrogen–donor–monomer systems.

We are indebted to Academician A. N. Terenin and to B. A. Dolgoplosk and Kh. S. Bagdasaryan for discussions. *Translated by* J. E. S. Bradley

* Measurements on chlorophyll or pheophytin (10^{-4} M) plus ascorbic acid (10^{-2} M) in pyridine illuminated directly in the evacuated cavity of an e.p.r. apparatus gave a signal which vanished as soon as the light was cut off. These experiments were done on the apparatus at the Institute of Macromolecular Compounds, Academy of Sciences of the U.S.S.R. We are indebted to Professor S. E. Bresler for his co-operation in these measurements.

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* There exists a translation into English of this work.

ULTRA-VIOLET FLUORESCENCE BY PYRIDOXIN, INDOLE AND THEIR DERIVATIVES*

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SEVERAL studies have been made recently of the ultra-violet fluorescence of biological compounds which do not fluoresce appreciably or at all in the visible region [1–3], including solutions of aromatic amino acids, proteins, nucleic acids and vitamins. Some of these fluoresce in the ultra-violet. Fluorescence spectra recorded from microcrystals are reported here and compared with the fluorescence spectra of the solutions. Pyridoxin, indole, and certain derivatives of the latter were used; these show fluorescence maxima in the near ultra-violet.

METHODS

An ultra-violet microscope (Brumberg) fitted with a special spectrograph [1–4] was used; an enlarged image of the microcrystals and the corresponding absorption or fluorescence spectra was recorded on the plate. The exciting radiation was

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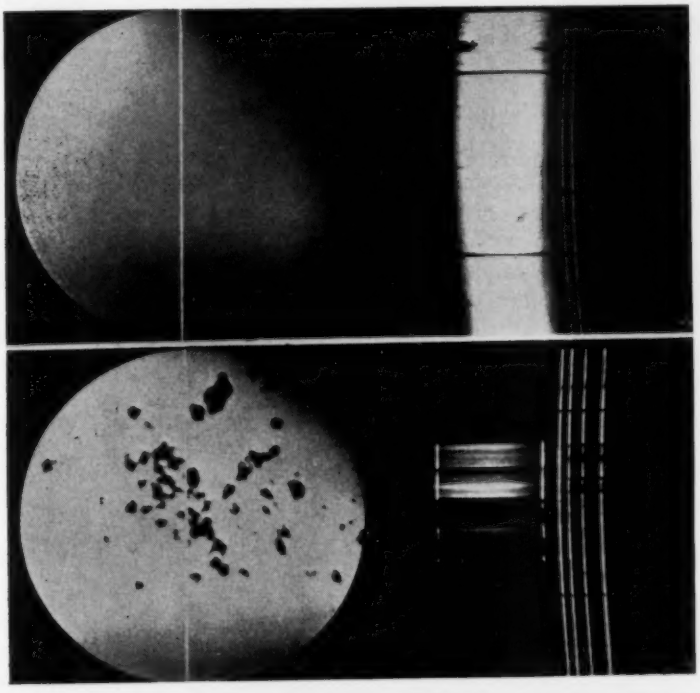

FIG. 2. Fluorescence spectrograms of (*a*) indole crystals, (*b*) aqueous indole.

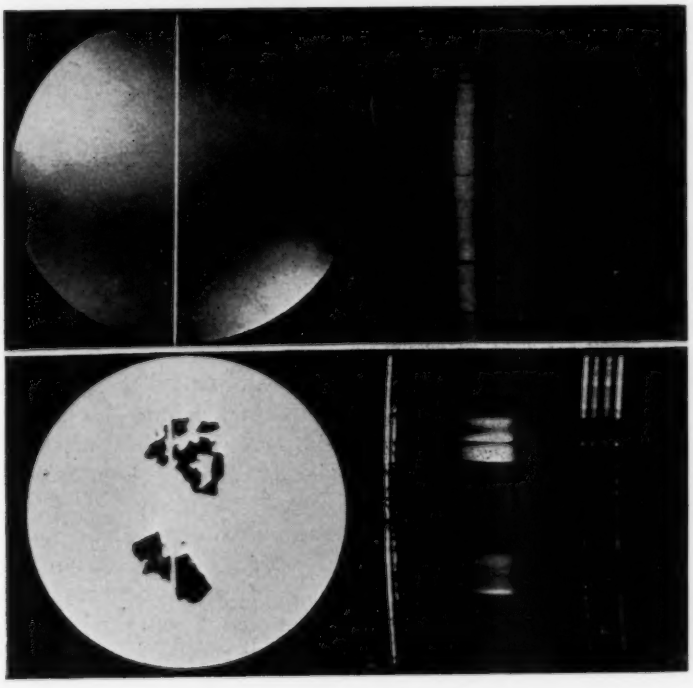

FIG. 1. Fluorescence spectrograms of (*a*) pyridoxin crystals, (*b*) aqueous pyridoxin.

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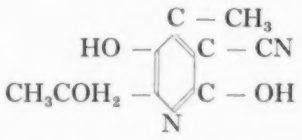
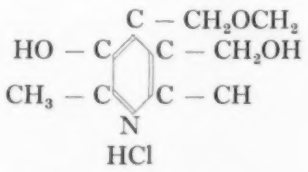
focused on the specimen using an aluminized mirror, a quartz condenser and a quartz–fluorite achromatic objective. The ×10 objective was of N.A. 0·2. A SVD-120 A mercury arc was used, with a filter consisting of gaseous bromine and chlorine to separate out the 250–280 mμ range. The crystals were held between a quartz slide and a quartz coverslip; a dark-field stop was used to reduce the exciting light entering the spectrograph, to which end a WG-7 filter (Schott) was also inserted; the transmission curve of the filter was allowed for in calculating the curves. "Astro" type plates were used, as being of high sensitivity in the ultra-violet; the intensities were determined from the blackening, using an MF-4 microphotometer and the characteristic curves for the emulsion. Figures 1(*a*) and 2(*a*) (lower sections) show fluorescence spectrograms for pyridoxin and indole crystals respectively; the upper sections show images taken at 250–280 mμ. The slit traces intersect the images and show which parts of the crystals were used to record the spectra shown in the lower sections.

The materials used were pure crystals of pyridoxin HCl, ethylacetal pyridoxal HCl, pyridoxamine di-HCl, 2-methoxymethyl-4-methyl-5-cyano-6-hydroxypyridine, of structure



a pyridoxin derivative, the antivitamin of structure



indole, indolylacetic acid and indolylpropionic acid.

RESULTS

The results were represented as curves similar to that of Fig. 3, which shows fluorescence intensity (in arbitrary units) vs. wavelength for pyridoxin. The fluorescence maxima are recorded in Table 1, accurate to ± 5 mμ; the values for pyridoxin, pyridoxal and pyridoxamine virtually coincide. The pyridoxin isomer and the antivitamin show identical long-wave shifts; pyridoxin and its isomer showed the brightest fluorescence, and the sharpest maxima. The other materials showed weaker fluorescence and flatter maxima; indole and its propionic acid derivative fluoresce fairly weakly (maxima at 330 mμ), while the derivative of acetic acid fluoresces more brightly, with a steeper curve and a maximum at longer wavelengths (360 mμ). The fluorescence intensities are only rough, because transmitted light is used, and no allowance can be made for scattering or reabsorption of the fluorescent emission. The same objective was used throughout, and closely similar, thin crystals selected, so far as possible, in order to get comparable results.

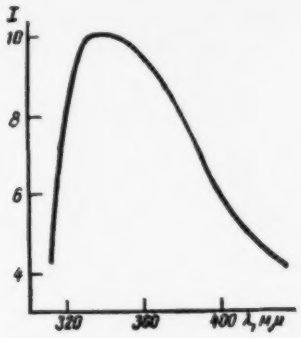


FIG. 3. Fluorescence spectrum of pyridoxin crystals.

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Solutions were also used with the microscope, although other types of apparatus [1–3] would have been more convenient. The quartz coverslip and slide were replaced by special cylindrical quartz cells 5 mm in diameter; the technique was otherwise unchanged. In all cases the solutions were at pH 7 in 0·1 M phosphate buffer (Table 1). Figure 4 shows the fluorescence curve for indole in solution, as an example.

The long exposures required (40–50 min) are somewhat of a disadvantage, as many organic compounds (e.g. amino acids) may thereby be damaged, particularly if the irradiations are in air. A partial test for whether such damage had occurred was carried out as follows. The crystals were placed in a special metal cell, fitted with quartz windows, which was evacuated by a rotary pump during the exposure; no noticeable change in the spectra resulted. Using a pyridoxin solution, several successive exposures were taken, each with the normal 50 min exposure; no effect was found.

Other workers have also examined pyridoxin, pyridoxal, pyridoxamine and indole in solution [1]; the published maxima agree with ours. No data have previously been given on the fluorescence of the crystals. The results will be used in localizing the compounds in biological objects.

The author is indebted to Professor M. N. Meisel' and Dr. E. M. Brumberg for guidance in this work.

TABLE 1. FLUORESCENCE MAXIMA OF CRYSTALS AND OF SOLUTIONS AT pH 7 (mμ)

Compound	Maxima		Concentration of aqueous solution
	Crystals	Solutions	
Pyridoxin	340	405	10^{-5}
Pyridoxal	340	370	10^{-5}
Pyridoxamine	330	405	5×10^{-6}
Pyridoxin isomer	410	375	10^{-5}
Pyridoxin antivitamin	400	410	10^{-5}
Indole	330	360	3×10^{-6}
Indolylacetic acid	360	365	4×10^{-6}
Indolylpropionic acid	330	360	4×10^{-6}

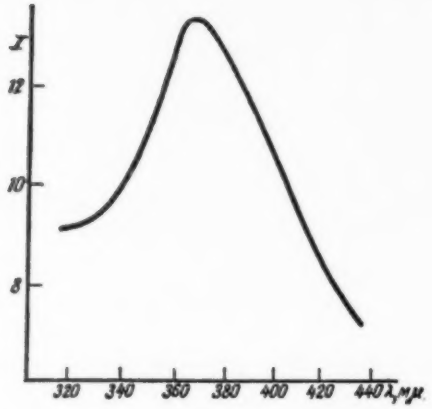

Fig. 4. Fluorescence spectrum of aqueous indole.

SUMMARY

(1) The near ultra-violet fluorescence of certain microcrystals has been studied using an ultra-violet microscope fitted with a spectrographic attachment. Fluorescence spectra from crystals of pyridoxin and some of its derivatives, and from indole and two of its derivatives, are reported.

(2) The same method has been used with solutions of the above materials.

Translated by J. E. S. Bradley

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THE MECHANISM OF THE PHOTODYNAMIC EFFECT*

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It has been shown repeatedly that visible light has a damaging effect on biological objects when dyes are present (photodynamic effect). It has also been shown that

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oxygen is always involved at some stage [1]. Several mechanisms have been proposed, of which Blum's [2] is the soundest and most widely accepted:

$$K + h\nu \rightarrow K^*$$
$$K^* + M \rightarrow M^* + K$$
$$M^* + O_2 \rightarrow MO_2$$

where K is the photosensitizer dye, M the substrate and MO_2 its oxidized form; stars denote excited states. Here the oxygen oxidizes the substrate, previously activated by collision with excited sensitizer. The oxygen must of course be present at the time of illumination, since the excited-state lifetimes are small. It is therefore considered that the separate stages (sensitizer activation, energy transfer to the substrate, and reaction of the latter with oxygen) cannot be distinguished—see for example a recent review [2, p. 698]. We consider this opinion wrong, since long-lived excited states can exist in protein and other macromolecules [3]; these states have been found to be produced by ionizing radiation [4]. Myosin has been shown not to lose its enzyme activity, but to go over to an excited state which persists for hours or days, when irradiated. Inactivation only occurs when oxygen is admitted to the excited protein. The physical nature of this excited state is at present unknown. The radiation energy stored in such states may be the cause of radiobiological after-effects.

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We give here proof that dye-sensitized protein molecules may go over to long-lived excited states when illuminated, and that the effects of oxygen are analogous to those found with ionizing radiations. Myosin was the protein and methylene blue the sensitizer. The myosin, prepared from rabbit muscle by Lyubimova's method, was dissolved in 0·5 M KCl to give 0·63 mg/ml and methylene blue added; the solution, placed in a glass cell in water at 3–4°C was illuminated from both sides by 300 W filament lamps fitted with reflectors. The photodynamic effect was determined from the fall in ATPase activity; with the dye at 2×10^{-5} M (6×10^{-3} mg/ml) the dark effect was a reduction in activity of not more than 10 per cent. There was of course no effect with no dye present; the inactivation followed a nearly exponential law when dye was present (Fig. 1). Even very prolonged illumination (over 3 hr) caused no

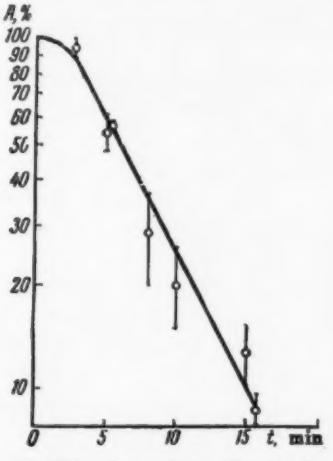

FIG. 1. Activity of myosin vs. illumination time.

inactivation if the oxygen was first removed using a rotary vacuum pump; here doses greatly exceeding those which completely inactivate the protein in aerated solutions are well tolerated. This agrees with other evidence [5] that oxygen is required to produce the photodynamic effect with myosin.

It is not essential that the oxygen be present during the illumination, however; the protein is inactivated even if stored in an air-free state for several hours before oxygen is let in. The sudden activity change was observed as follows. A cell which had been irradiated when oxygen-free and stored in the cold was opened and several identical protein samples taken; half of these were incubated with ATP and other reagents saturated with oxygen, and half in oxygen-free conditions. The activities found in the first half were much lower. The myosin dropped in activity as oxygen was taken up, and the level found when excess oxygen was present at the start was gradually approached (Fig. 2). The excited state thus lasts a long time in this system, and oxygen inactivates by reacting with the excited state. The activity falls off uniformly with dose at low doses, but remains virtually constant at about 75 per cent of the initial activity at high doses (Fig. 3).

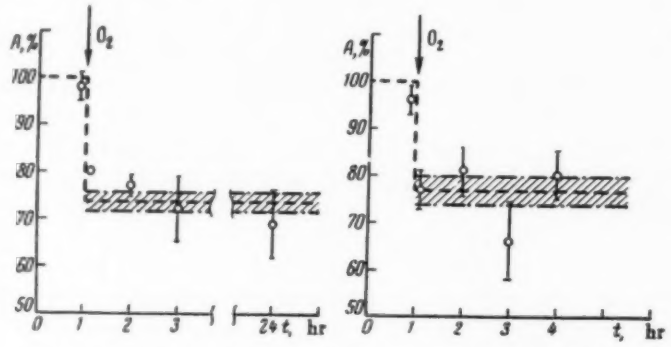

Fig. 2. Activity changes in myosin after anaerobic illumination followed by oxygen admission (denoted by arrow). (*a*) 15 min illumination, (*b*) 1 hr; *t* is time after end of illumination. The hatched area shows the statistical error of the mean.

The excited state was shown to reside in the protein and not in the dye by adding dye solution to protein solution previously irradiated for a long time; no fall occurred (activity 105 $\pm$ 7 per cent). Any possible oxygen effect on the dye was eliminated by illuminating it and adding the product to the oxygen-free protein; no effect was found (activity 98 $\pm$ 4·5 per cent). The substrate, and not the dye, stores the excitation. The effect is similar to that found with oxygen and ionizing radiations, where there is no sensitizer. A choice can thus be made between the various mechanisms which have been put forward. Let us consider three mechanisms essentially different from Blum's, to which almost all other mechanisms are in fact equivalent (I is Kautsky's, II is the Warburg–Bach–Engler, and III is Wieland's).

I	II	III
$K + h\nu \rightarrow K^*$	$K + h\nu \rightarrow K^*$	$K + h\nu \rightarrow K^*$
$K^* + O_2 \rightarrow K + O^*_2$	$K^* + O_2 \rightarrow KO_2$	$K^* + HM \rightarrow KH + M$
$O^*_2 + M \rightarrow M_{om}$	$KO_2 + M \rightarrow M_{om} + K$	$KH + O_2 \rightarrow K + H_2O$

In I the substrate is damaged by excited oxygen, in II by an oxidized form of the dye. Our results agree with neither of these, because the excited-state lifetime of the dye is short compared with the storage times used, and oxygen inactivates after prolonged storage in the dark. In III the excited dye oxidizes the substrate, while the oxygen restores the dye to its initial state. If III was correct we should have found inactivation in the oxygen-free specimens, but this was not so.

The oxygen reacts with some excited state of myosin; this agrees in a general way with Blum's mechanism, but the ideas require much detailed revision. K* denotes an excited state of the dye (probably a viradical triplet state [6]) with a lifetime not exceeding a second or so. M* denotes an excited state of the substrate, also short-lived. Our data show that the excited state in myosin can be retained for a long while (perhaps as long as may be desired). The effect occurs somewhat as in Blum's scheme, if M* is taken as this latter type of state. The last stage (oxygen action) can then be remote in time from the others.

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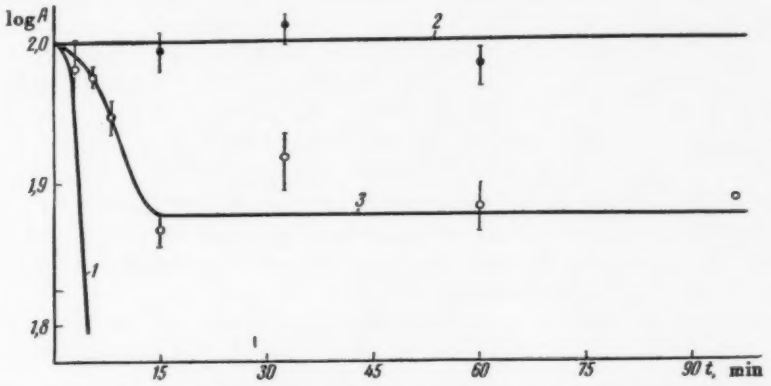

FIG. 3. Activity of myosin vs. illumination time for aerobic conditions (1), anaerobic (2), and anaerobic followed by oxygen admission (3).

In all cases the inactivation produced by admitting oxygen after the irradiation was less than when oxygenated myosin was irradiated; beyond a certain dose no further effect occurred. There are three possible causes for these effects; a final choice can only be made from future experiments. They are as follows.

(1) The mechanisms of the simultaneous and subsequent oxygen effects are the same (reaction with excited molecules); all the states are long-lived, but oxygen can react with only some of them after irradiation, for some reason.

(2) The two mechanisms are the same, as above; the differences are due to there being both long-lived and short-lived states, of which the latter require the oxygen to be present during irradiation. Here the mechanism is partly as in Blum's scheme, and partly involved long-lived states.

(3) All the excited states are long lived, but the effects of oxygen during and after illumination are different. During the illumination the oxygen can react additionally with the substrate, either because it is itself activated, or because it gives active products.

The inadequacies of the mechanisms so far proposed can therefore to some extent be referred to different actions of oxygen present during or after irradiation. Future work will show whether Blum's scheme is applicable at all, or whether all the excited states are long lived. Our data support the first proposal; if the absorbed energy is supposed to be redistributed in the protein, while the oxygen can react only with certain parts of the molecule, we can explain why the subsequent inactivation (about 25 per cent) is independent of the dose.*

This mechanism has been demonstrated only on myosin, but there are reasons for supposing that it occurs in many other cases, as the main substrates are usually proteins or proteids [2]. These can store energy for long times, so the radiation after-effects found with many proteins probably imply that the mechanism involving a long-lived state which then reacts with oxygen is a common one. The degree of inactivation by oxygen of course depends on the individual properties of the protein. The methods must be adequate for the purpose if oxygen effects after illumination are to be detected; lack of proper methods explains why oxygen after-effects were not detected earlier.

The situation has been made somewhat more clear by some comparisons between the effects found in aerobic and anaerobic conditions [7–13]. The full details of the methods used cannot always be recovered from the published papers, but it is clear that in some of the experiments it would have been difficult or impossible to detect the effect. In erythrocyte photohaemolysis oxygen absorbers were used to produce anaerobic conditions [7], and these absorbers remained after illumination; or else evacuated cells were used, with all subsequent spectral analyses done in oxygen-free conditions [8]. Live frogs [9] were examined for behaviour changes before transfer from anaerobic to aerobic conditions, etc.

In some old experiments on enzyme photo-inactivation the methods were such that the oxygen after-effect could not be detected since the activity changes after cutting off the light were not examined. In the photoreactions of human skin [11] or of muscle contraction [12, 13] it is difficult to anticipate that oxygen would produce any special damage after illumination. Protection by anoxia has been observed only qualitatively in photoinactivation of bacteria [10]; the difference between the controls and those irradiated anaerobically and then exposed to oxygen was not established. The published experiments do not therefore contradict our view that the mechanism of the photodynamic oxygen effect is of common occurrence.

Translated by J. E. S. Bradley

* If the cause were that the light caused potential damage in 25 per cent of the molecules, the fraction damaged should increase with the dose, which is not found. We must therefore suppose that the oxygen can inactivate only one quarter of the enzymatically active centres in the molecule after irradiation.

More concentrated solutions (4 mg/ml) give similar results; the after-effect is as before. The number of active centres in the myosin molecule is not yet reliably established. Our data show that there must be at least four (or in general $4n$).

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THE DELAY TIME OF THE ACTION POTENTIALS FROM THE GIANT NERVE IN THE SQUID AND FROM THE SCIATIC NERVE IN THE FROG*

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(*Received* 26 *February* 1958)

Much work has been done on the rates of physiological reactions, which play a major part in muscle physiology. Little has been done on the excitation aspect, however, particularly as regards the sequence in time of the electrical reactions in the nerve. Bishop [1] found that the spike produced in the sciatic nerve of the frog by an induction-coil shock of maximum strength started some 60 μ sec after the start of the stimulus. Blair and Erlanger [2–3] used a phalangeal frog preparation, and determined the delay time using d.c. and induction coil stimuli.† The delay time fell as the stimulus became stronger, in a way similar to, but not the same as, the way the duration of the spike increased with the stimulus strength; also the delay was much longer than the duration of the stimuli provided by coil shocks, so it is not simply a function of stimulus duration. Similar results have been obtained with single nerve fibres [4–8]. As regards threshold stimuli, the published data only indicate that the delay drops by 10 per cent when the duration of the coil shock is reduced by a factor of two [2], and that the delay is a function of duration [7–8]. Threshold stimuli of varying durations were used in the present studies on the delay time.

METHODS

The sciatic nerve in *Rana temporaria*, and the giant nerve in the squid *Ommatostrephes sloanei pacificus* were used. A chronaximeter used in the *RC* range 1 μsec–5 msec, or square pulses of duration from 20 μsec to 4 msec, were employed,

* Biofizika **3:** No. 5, 568–575, 1958.

† They termed the time from the start of the stimulus to the start of the response the make–response time, and the time from the end of the stimulus to the start of the response the break–response time. Others have termed these times latent periods; as " latent period " usually denotes the time from the end of a stimulus to the start of the response, we think it better to call the time from the start of the stimulus to the start of the response the delay time. We use this term in dealing with the published data.

with the negative side earthed. The repetition rate was 25 c/s. Mozhaeva's method [9] was used to record at the site of the stimulus, using one input only of the balanced amplifier; the other input was fed via a high-resistance, integrating circuit (adjustable) from the stimulator. The earth lead was then common to both circuits. The action potentials were recorded on an oscilloscope. The delay time was determined directly from the screen.

The sciatic nerve was placed on silver electrodes (not silver chloride) and immersed in Ringer except during measurements. The single nerve in the squid was placed on a network of platinum electrodes in a humidified chamber.* In all cases the temperature was 20–22°C. Other special features of the methods will be dealt with in the following text.

RESULTS

(1) *Giant nerve of the squid.* The delay times found with threshold stimuli of varying durations were studied on 15 fibres, using condenser discharges with 3, and square pulses with 12; the results were the same in both cases. Figure 1 shows the results from one experiment, in which the threshold stimulus frequency was 25 per sec; 8–10 traces were recorded at each stimulus. The delay times varied considerably; no such fluctuations occurred with larger stimuli. The spikes occurred during the

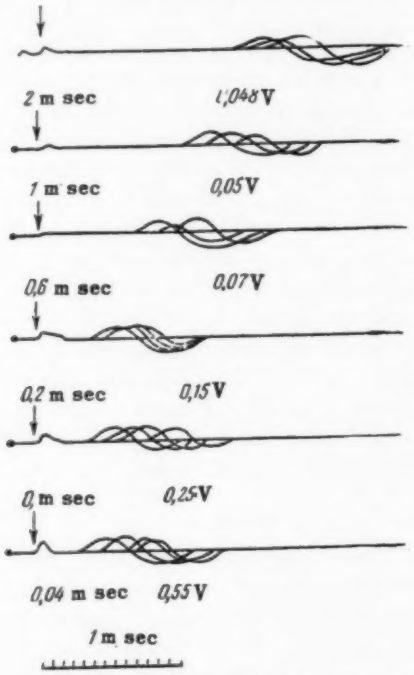

FIG. 1. Delay time of spike vs. duration of threshold stimulus. From the experiment of 2 October 1957 at 21°C. The magnitudes and durations of the stimuli are given on the curves. The arrow denotes the start of a stimulus.

* The method used has been described in detail [10].

stimuli only in the 2 msec case; in the other cases it was delayed till the stimulus had finished. All the fibres showed the same effect. The results were treated statistically; the arithmetic means from eight experiments are given in Table 1. The largest and smallest values were used as the delay times varied. The least delay was 1·24 msec, and the longest 1·50 msec, when 2 msec stimuli were used. The data of Table 1

TABLE 1. SPIKE DELAY TIMES AND TIMES FROM THE END OF THE STIMULUS TO THE BEGINNING OF THE RESPONSE FOR SQUID GIANT NERVE (Arithmetic mean values).

Stimulus duration (msec)	Threshold (V)	Spike delay (msec)		Time from end of stimulus for least delay time (msec)
		Maximum	Minimum	
2	0·049 ± 0·005	1·50 ± 0·09	1·24 ± 0·07	−0·76
1	0·059 ± 0·006	1·15 ± 0·06	1·03 ± 0·05	+0·03
0·6	0·072 ± 0·009	0·89 ± 0·05	0·68 ± 0·03	+0·08
0·2	0·13 ± 0·01	0·65 ± 0·05	0·42 ± 0·03	+0·22
0·1	0·21 ± 0·02	0·55 ± 0·05	0·34 ± 0·04	+0·24
0·04	0·50 ± 0·05	0·53 ± 0·04	0·33 ± 0·03	+0·29

give a useful time of about 1·5 msec (the time cannot be determined precisely). Hence, if threshold stimuli of duration longer than the useful time were used, the spike occurred at about the useful time after the start. The delay tends downwards to a limit as the stimulus duration is reduced; the values for 100 and 40 μsec stimuli are practically the same. The time from the end of the stimulus to the start of the response is also given.

The relation of delay time to stimulus strength could not be studied because strong stimuli could not be balanced out properly; the start of the spike could not be determined at stimuli 5–10 times threshold, though the changes in the maximum could be followed. Five experiments were done with stimuli lasting 40 μsec. The

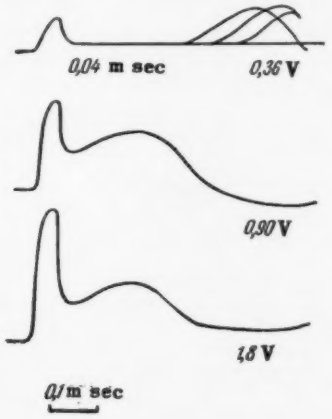

FIG. 2. Delay time of spike vs. stimulus amplitude. From the experiment of 27 September 1957 at 21°C. Stimulus duration 0·04 msec. The amplitudes are given on the curves.

delay time decreased on increasing the stimulus and tended to a limiting value shorter than that found with threshold stimuli. Figure 2 shows the results for one experiment; the change between 0·36 V and 0·9 V is large, but scarcely any further change occurs at 1·8 V.

The response was found to diminish somewhat when long (1–2 msec) threshold stimuli were used; measurements were made only in 5 experiments, as photographic recording was not always used. Taking the spike at 100 μsec as 100 per cent, the 2 msec value is 67–86 per cent; Mozhaeva [11] has found the same effect with the sciatic nerve in the frog.

(2) *Frog sciatic nerve.* Exact delay time measurements on multiple preparations were complicated by statistical factors; since the time when the maximum spike occurred could not be determined, we studied only the minimum spike.

TABLE 2. SPIKE DELAY TIMES WITH THRESHOLD STIMULI OF VARIOUS DURATIONS. FROG SCIATIC NERVE. MEAN VALUES FROM 11 EXPERIMENTS

RC (msec)	Threshold (V)	Spike delay (msec)
5	0·49 ± 0·04	1·43 ± 0·08
1·82	0·56 ± 0·04	1·19 ± 0·05
0·182	1·58 ± 0·10	0·58 ± 0·04
0·0195	9·5 ± 0·8	0·32 ± 0·02
0·01	17·4 ± 1·2	0·30 ± 0·01
0·0056	31 ± 2	0·30 ± 0·01

This was about 0·1 of the maximum one in our experiments. The delay times were measured with condenser discharges, with *RC* varying from 5·6 μsec to 5 msec. The normal shape of the strength–duration curve indicates that the threshold in each case corresponds to the same response. Table 2 gives the mean values from 11 experiments. Just as with the squid, the spike occurs during the stimulus during long (rheobase) stimuli, on average after 1·43 msec, and the delay about equals the useful time. At $RC = 10$ and 5·6 μsec the spike occurs after 0·3 msec, i.e. the delay shortens with the duration, down to some limit. The limit is about the same as others have found for short threshold stimuli. Our rheobase results agree well with the published data, which are collected in Table 3.

TABLE 3. SPIKE DELAY TIMES WITH THRESHOLD STIMULI OF VARYING DURATION

Object	Type and duration of stimulus	Spike delay (msec)	Reference
Frog phalangeal preparation	D.C.	1·26–1·83	[3]
Frog phalangeal preparation	Induction shock, 40 μsec	0·20–0·40	[2]
Frog phalangeal preparation	Condenser discharge, $RC = 4{\cdot}3$ μsec	0·156–0·268	[12]
Single frog myelin fibre	Square pulse, 5 μsec	1·8	[8]
Single frog myelin fibre	Square pulse 7 μsec	0·3	[8]
Single toad myelin fibre	D.C.	2·1	[6]
Frog sciatic nerve	Condenser discharge, $RC = 5$ msec	1·43	Own data
Frog sciatic nerve	Same, $RC = 5{\cdot}6$ μsec	0·30	Own data

(3) *Changes in delay time when the sciatic nerve is altered.* The identical behaviour shown by the giant squid nerve and by the sciatic nerve fibre in the frog indicates that one is justified in operating with an intact nerve trunk; we therefore used altered frog nerves to study delay time changes. The studies on whole nerves were of course somewhat approximate, since the spike heights might have changed, and no account was taken of this. The effects of storage in Ringer and of treatment with 0·5% sodium citrate or 0·3% $CaCl_2$ (both isotonic) were studied. Table 4 gives the results.

TABLE 4. THRESHOLD AND SPIKE DELAY CHANGES PRODUCED IN FROG SCIATIC NERVE

Treatment	No. of experiments	Stimulus duration (msec)	Threshold change (%)	Delay time change (%)
2·5 hr in 0·5% sodium citrate	12	5	−71±3	+10±3·4
		0·0056	−48±4	+8±2·2
24 hr in Ringer	11	5	+100±24	−39±5
		0·0056	+34±10	−13±2
2·5 hr in 0·3% $CaCl_2$	16	2	+210±17	−8±2·3
		0·2	+110±10	0±3·7

The threshold and delay time are given for the shortest and longest stimuli, for brevity. The sodium citrate caused the threshold to drop sharply in all cases, while the delay tended to increase; the Ringer raised the thresholds and shortened the delay, while the $CaCl_2$ raised the thresholds, shortened the delay for 2 msec stimuli and did not alter the delay for 0·2 msec. stimuli.

The data clearly indicate that the delay did not vary much; the changes found in rheobase stimulation showed the same trend as that found in the useful time (Ringer and $CaCl_2$ shortened this, while citrate increased it). No definite conclusion can be drawn for short stimuli, since the changes found were variable.

DISCUSSION

It might be supposed that the spike should occur as soon as the threshold stimulus ceases (as in the condenser theory of the 1930s), or after a fixed delay; but neither occurs, since the delay time decreases with the stimulus duration, though not in direct proportion. The electrical stimulation mechanism should really be known in order to discuss the data properly, but so far no proper studies have been made of the tissue processes involved. Blair and Erlanger [2] suppose that the stimuli charge some unphysiological capacitance which then discharges physiologically, and that this itself constitutes the local excitatory state. Rosenblueth [13] inclines to another view on the intermediate process. Hodgkin [5] states that some local subthreshold reaction occurs in the time between the start of the stimulus and the start of the spike. Hodgkin and Huxley's theory [14] indicates that the delay time must depend on the membrane depolarization rate, and hence on the permeability of the membrane to sodium ions. Nasonov's denaturation theory relates the delay to the denaturation rate, and to the rates at which the diffusion and phase potentials are set up. The

precise mechanism being unknown, it is difficult to explain why the delay varies with the duration for threshold stimuli. The delay decreases the more rapidly the threshold dose is delivered; the final result clearly depends on whether the spike is caused by some reaction that begins to develop at the start of the stimulus, or by one that only goes to completion when the stimulus is over. The reduced spike height in rheobase stimulation supports the latter idea. Further studies using stimuli other than threshold stimuli of varying durations are required to elucidate the matter.

Tasaki [6] states that the strength–delay relation is described by the Horweg–Weiss equation; our data are inadequate to show if this is so, but they do indicate that a limit to the delay time occurs as the strength is increased. It is difficult to see how the delay could become as small as the equation $i = (a/t) + b$ would imply (t = delay time, i = threshold current, and a and b are constants). Tosaki's results derive from two rheobase strengths; the equation probably only applied over a small range of strengths.

We determined the spike delay directly, although the method is complicated. It is natural to seek a stimulus parameter which is closest in magnitude and kind to the delay time; Blair and Erlanger [3] have shown that rheobase stimulation gives a delay time roughly equal to the useful time and decreasing with the stimulus strength. The only threshold stimulus which gives a daily time equal to the stimulus time is that of duration the same as the useful time. No satisfactory explanation for the useful time can at present be given, and no determining cause for the delay time can be stated. There is, however, no real obstacle to using the useful time as an index of the delay time, though rheobase stimulation lasts some 5–10 times as long as the "natural stimulus", i.e. the rising phase of the spike. How the delay time alters with duration of threshold stimulus in the treated nerves has not yet been properly explained, but, in any case, measurements of useful time give reliable data on delay times in rheobase stimulation. The fresh data confirms the views expressed by leading physiologists early in this century; thus Hildemeister proposed to measure the "Hauptnutzzeit" (main useful time) to determine reaction rates. Lucas introduced the chronaxy and excitation time concepts, from the idea that the excitation occurred the more rapidly the shorter the useful time, and that it was in practice more convenient to determine the excitation time from the rheobase than from the useful time. This is, in our opinion, why chronaximetry became widely used, though the importance of the chronaxy as the easily determined analogue of the useful time was unfortunately forgotten with the passage of time. Several descriptions of what is in our view an artificial correlation between the chronaxy and the duration of the rising phase of the spike have been given; this divorce of the chronaxy from its general strength–duration relationship has led to many misunderstandings and errors, as the discussion on chronaximetry caused by Nasonov and Rosental's paper [15] shows. Of all those who participated, only Nasonov and Rosental' stated that the chronaxy was closest in meaning to the useful time. However, we have shown above that, when the stimulus duration equals the chronaxy, the delay time is much larger than the discharge time, and so the chronaxy cannot be used to indicate the spike delay. Many who have determined the excitation time or other times write of rates of excitation, rates of physiological reaction, etc. The use of such all-embracing terms produces misunderstandings and is unjustified, particularly as excitation often only means action potential in such cases. It would

always be better to state the rate of what process was studied, or what particular time was measured.

It is possible to estimate spike rise times in rheobase stimulation by measuring the useful time.

SUMMARY

(1) The spike delay–time at the stimulation site has been studied on the sciatic nerve of the frog and on the giant nerve of the pacific squid.

(2) The time has been measured for threshold stimuli of various durations; with durations varied from 5 msec to 5·6 μsec the average delay varied from 1·43 to 0·3 msec in the sciatic nerve of the frog; the squid nerve gave values of 1·24 to 0·33 msec for a range from 2 msec to 40 μsec.

(3) Rheobase stimulation gives a time equal to the useful time; the time falls with the stimulus duration, but not proportionately; a limit is approached for stimuli shorter than 100 μsec.

(4) Storage in Ringer, and treatment with sodium citrate or $CaCl_2$ are studied for their influence on the delay using frog sciatic nerve. The changes are slight even when the curve of strength vs. duration is greatly altered.

(5) It is suggested that the spike delay–time can be determined from the useful time in rheobase stimulation.

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* There exists a translation into English of this work.

EXOSMOSIS FROM PLANT CELLS AFTER X-IRRADIATION*

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Exosmosis is a very important index of the vitality and vital activity of a cell, and is one aspect of selective cellular permeability; radiobiologists have long given it special attention [1]. No definite results have yet been given for plants, however, and the published data are very conflicting. It has been found [2] that 250 r X-ray doses to oat plants caused no change in root-tip exosmosis, while 500 r doses produced a 52 per cent increase, and 13 kr, 100 per cent. Spring wheat seedlings given 1 kr and 10 kr, and then placed in distilled water in the first 30 min eliminated 3—4 times as much radiophosphorus as did controls [3], but this difference soon vanished. Pieces of potato tuber exosmosed more electrolytes after irradiation, but the effect was dose-independent, even in the 3–100 kr range [4]. Our work was directed to this problem, and special care was taken to determine the exosmosis immediately after irradiation, as well as at later times, until clear signs of radiation damage appeared.

GENERAL EXOSMOSIS FROM PLANT CELLS IMMEDIATELY AFTER IRRADIATION

Two wheat varieties, Spring Moskovka and Winter 599, both superelite selections from the Wheat Industry Institute, were used. Small intact seedlings and roots were used, with two methods of determination: interferometry and evaporating extract samples to dryness.

Exosmosis from intact seedlings. Seeds of identical size were germinated and seedlings of the same size selected for irradiation. A RUM-3 unit was used at 180 kV, 10 mA, no filter, with a dose rate of 418 r/min. The seedlings were then placed at once in distilled water for 3 min, and then placed in water for 1–1·5 hr; the baths were kept agitated on a shaker to aid exosmosis. The water was then run off and the content of exosmosed material determined. Seedlings killed by boiling in water were also used, to compare the effects of irradiation and of high temperatures on exosmosis (Tables 1 and 2).

In every case the germination conditions, seedling size, etc., varied somewhat, so the results can only be compared within experiments. The same general results were always found, though; the exosmosis varied little during the first few hours after irradiation. Increasing the dose from 3 to 30 kr had no appreciable effect, but boiling had a large effect, as would be expected; the dead seedlings showed very much more exosmosis.

Exosmosis from roots. The seeds were germinated on tap water and sets of 100 seedlings of identical size were laid out on waxed metal grids on Koch flasks filled

* Biofizika **3**: No. 5, 576–581, 1958.

TABLE 1. GENERAL EXOSMOSIS FROM MOSKOVKA SPRING WHEAT SEEDLINGS

Dose (kr)	Interferometer Readings		
	Control	Irradiated	Difference
3	0·660 ± 0·010	0·632 ± 0·008	0·028 ± 0·013
Repeat measurement*	0·565 ± 0·002	0·569 ± 0·002	0·004 ± 0·003
3	0·769 ± 0·002	0·859 ± 0·003	0·010 ± 0·004
3	1·246 ± 0·020	1·265 ± 0·020	0·019 ± 0·028
30	0·660 ± 0·010	0·679 ± 0·004	0·019 ± 0·009
Repeat measurement	0·565 ± 0·002	0·562 ± 0·003	0·003 ± 0·004
	Seedlings killed by boiling		
	Live	Killed	Difference
	1·184 ± 0·002	4·477 ± 0·260	3·293 ± 0·260
	1·040 ± 0·004	5·506 ± 0·021	4·466 ± 0·021

* The repeat was done at once after the first, in exactly the same conditions.

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with tap water, with the roots in the water and the seeds, shoots and grid above it. The flasks were placed in a lumistat until the time for irradiation (2–5 days); the water was changed each day. The plants and grids were removed and the roots well washed with distilled water before irradiation; the grids were turned over to present the roots directly to the X-ray tube and placed on top of the Koch flasks, to prevent drying out of the roots. The RUM-3 unit was used as above. The controls were treated

TABLE 2. GENERAL EXOSMOSIS FROM 599 WINTER WHEAT SEEDLINGS*

Dry residue, mg/g of dry seedling weight		
Control	Irradiated	Difference
1·35 ± 0·08	1·28 ± 0·04	0·07 ± 0·09
3·63 ± 0·10	3·53 ± 0·20	0·10 ± 0·22
3·38 ± 0·08	3·02 ± 0·15	0·36 ± 0·17

*All doses 1 K.R.

in exactly the same way, apart from irradiation. All plants were placed in Petri dishes containing precisely known amounts of distilled water which were then placed in the lumistat for fixed times. The water was then drained off, evaporated to dryness, and

TABLE 3. GENERAL EXOSMOSIS FROM 599 WINTER WHEAT ROOTS

Dose (kr)	Dry residue per 100 seedlings (mg,)		
	Control	Irradiated	Difference
1	7·20 ± 0·80	6·20 ± 0·70	1·00 ± 1·06
3	2·27 ± 0·37	2·65 ± 0·28	0·38 ± 0·46
5	15·30 ± 2·00	16·10 ± 1·30	0·80 ± 2·16

the dry residue weighed. Each test was done six times (Table 3). The result is the same; doses of 1, 3 and 5 kr produce no effect. The differences do not exceed the experimental error.

IMMEDIATE POST-IRRADIATION ELECTROLYTE EXOSMOSIS

Isolated undamaged roots were used in two series of tests. In the first series 4 day seedlings of Moskovka wheat were grown on distilled water in the lumistat; the roots of six sets of 150 were cut off and weighed. Two sets were given 300 r, two 3 kr, under conditions as in the earlier tests. The roots were placed in flasks containing identical amounts of distilled water and shaken for 1 hr. The water was drained off, fresh water run in, and the process repeated. The electrical conductivity values for the first two lots of water are given in Table 4. The same method was used in the

Table 4. Electrolyte exosmosis from Moskovka wheat roots

Dose (r)	Resistance (Ω)		
	Control	Irradiated	Difference
300	5775 ± 66	5842 ± 58	67 ± 88
Repeat	20,950 ± 565	21,125 ± 81	175 ± 571
3000	5775 ± 66	5455 ± 103	320 ± 122
Repeat	20,950 ± 565	20,225 ± 81	725 ± 789

second series, with 2 day 599 wheat seedlings of equal size, in sets of 80 laid out on perspex grids on Koch flasks filled with Mayer's solution. After 2 days on these grids the seedlings were irradiated at 180 kV, 15 mA, 374 r/min; the washing, etc., were as before. The Petri dishes contained 60 ml of distilled water, and were placed in the lumistat; the conductivities were determined after 3 hr (Table 5). The general results were as before; doses of 300 r, and 1, 3 and 5 kr gave no definite change in exosmosis.

Table 5. Electrolyte exosmosis from 599 winter wheat roots

Dose (kr)	Resistance (Ω)		
	Control	Irradiated	Difference
1	34,083 ± 2191	35,533 ± 1461	1450 ± 2634
20	34,083 ± 2191	34,833 ± 3919	750 ± 4490
5*	15,367 ± 1943	16,967 ± 866	1600 ± 2127

* Reading taken one day after irradiation.

ELECTROLYTE EXOSMOSIS 1 AND 3 WEEKS AFTER IRRADIATION

Here the object was to find how accumulated, unused growth materials [5, 6] affected the exosmosis. Leaves formed the best material; roots were unsuitable because they become covered in root hairs soon after irradiation, since the meristem zone is lost [7]. This would make conditions complex, since root growth is slowed down or stopped, while a large area of very active hair surface is formed.

Four-day 599 wheat seedlings grown on Mayer's solution using perspex grids and Koch flasks were irradiated at 180 kV, 15 mA, no filter, at 374 r/min and then replaced in the lumistat. After a week the leaves at the coleoptile level were cut off and divided into 2 g lots, which were put into flasks with 60 ml lots of distilled water; the flasks were shaken for 3 hr. The same was done with plants allowed to grow for 3 weeks (Table 6). The results were quite different from those found before. The

TABLE 6. ELECTROLYTE EXOSMOSIS AT 1 AND 3 WEEKS AFTER IRRADIATION

Dose (kr)	Time grown after irradiation (weeks)	Resistance (Ω)		
		Control	Irradiated	Difference
20	1	6832 ± 392	3785 ± 470	3047 ± 612
Repeat		19,450 ± 864	14,841 ± 1564	4609 ± 2430
5	3	5743 ± 512	2068 ± 104	3675 ± 522
Repeat		21,835 ± 1965	2978 ± 291	18,857 ± 1987

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plants were able to absorb minerals which they did not use up in growth (as this is completely suppressed by 5 and 20 kr doses [6]); these minerals therefore accumulated, and were exosmosed. This is particularly clearly seen after 3 weeks.

The following point is significant. The repeat tests shows a more marked exosmosis in the irradiated plants, although the material was by then partially depleted, particularly in the second test (3 weeks). Here the absolute exosmosis from the irradiated plants hardly changed between the first and second tests while the controls showed a very big change.

Exosmosis is not always increased by stored material though; some tests with 599 wheat illustrate this. Two-day seedlings were irradiated (500 r) and then grown on Knop's medium for 3 weeks. The irradiated plants grew, but somewhat more slowly than the controls.

The pattern normal to plants given 500 r was seen after the third leaf had grown; the leaf expanded rapidly and seemed to show that the plant was vigorous. The meristem cells were responsible for this, as they contract in volume and cause the extension zone to grow larger. The extension is caused by osmotically active materials, including electrolytes, entering the meristem zone. The youngest leaves in such plants were taken and extracts made as above, followed by two further extracts (one from living leaves, and one from boiled ones). The exosmosis times in the repeat determinations were in both cases reduced to 1 hr (Table 7). The irradiated

TABLE 7. ELECTROLYTE EXOSMOSIS FROM LEAVES SHOWING CELLULAR DISTENSION

Resistance (Ω)		
Control	Irradiated (500 r)	Difference
13,700 ± 579	34,317 ± 1685	20,617 ± 1278
Repeat using living leaves		
54,900 ± 900	110,000 ± 0000	55,100 ± 900
After boiling		
1617 ± 60	1882 ± 167	265 ± 177

plants showed much less exosmosis in the first and repeat measurements. The boiling greatly increased the exosmosis and made it almost the same for both groups.

DISCUSSION

X-ray doses of 0·3, 1, 3, 5, 20 and 30 kr have very little effect on exosmosis, the rate of which is practically the same in irradiated and control plants on the first post-irradiation day. The permeability, on which exosmosis depends, is thus also unchanged. Exosmosis is affected by cellular storage and use of materials, however. If minerals are accumulated and not used in growth they are exosmosed in large amounts. Leaves of plants irradiated a week previously exosmose more electrolytes than do ones from controls. The differences at three weeks after irradiation are even more marked. The same applies to the non-electrolytes (sugars). Sugars accumulate in irradiated plants supplied with minerals and air [6], and "sugar growth" (formation of drops of concentrated sugar solution in the leaves) can be observed. The sugar is exosmosed if too much accumulates.

The material used up by the cells must also be allowed for, though; osmotically active substances accumulate in vacuolized cells, but no exosmosis results, because the substances are used to draw water into the vacuoles. The substances are therefore exosmosed only when the cell has no further need of this osmotic "ballast".

These general aspects of cell permeability are very important to the effects of ionizing radiations on cells. The physiological processes of functional plant cells (all processes other than growth and division) are very radiostable; normal conditions are very little disturbed by doses of kiloroentgen magnitude.

SUMMARY

General and electrolyte exosmosis from wheat seedlings are used to show the following.

(1) Doses of 0·3, 1, 3, 5, 20 and 30 kr scarcely influence exosmosis.

(2) Exosmosis is much increased by stored materials not used up in growth.

(3) Material stored as the osmotically active content of vacuoles is not exosmosed.

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* There exists a translation into English of this work.

CLASSIFICATION OF THE DYNAMIC COMPONENTS IN THE CO-ORDINATION STRUCTURE OF LOCOMOTOR ACTS IN MAN*

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A CLASSIFICATION of the dynamic components in the co-ordination structure of locomotor acts in man is given here, in relation to walking and running. By dynamic components we mean the magnitudes and signs of the forces acting at the centres of gravity of the limbs, their phase relations, and the effects on them of various factors. The stability or otherwise of such components may indicate their roles; any classification must, in our opinion, start from this basis. Such classification may also be of both theoretical and practical importance, in that it may provide information on co-ordination of motion in general.

We have studied the changes in co-ordination occurring during walking in mountainous areas (see [1] for details and methods) and during running subject to fatigue, changes in distance run, and performance of extra movements (overcoming obstacles, jumping). Table 1 gives a general summary of the changes thus produced; the differences in co-ordination between athletes and untrained runners in essence define the changes occurring when a novice begins training. It is clear that the changes are very great, but certain major features can be distinguished.

Consider, for instance, the changes occurring in the centre of gravity of the leg of a trained but fatigued athlete running 400 m; these are shown in Fig. 1 in parametric form in terms of the longitudinal and vertical components of the forces at the centre of gravity during one full cycle. The left half is for no fatigue, at the start; the right when fatigue has set in at the finish. The changes are regular; the left half represents the cycle during normal running (for more details see [2], from which the notation derives). On the right, however, although we have the same kinks, several important changes have occurred. These are that the longitudinal component in the forward stride ($n\beta$) has lessened, while the vertical (C) has increased; the rearward phase (γ and D) shows falls. The reactive bending forces (Δ and E) and the forces driving the leg forward (ϵ) are not so well utilized at the joint. The braking forces (observed in leg straightening and denoted by nA in the vertical component) are much lessened, while the reactive forces (a) have increased.

This picture clearly corresponds to the running pattern seen in fatigue: the weakened backward kick, the leaden stride, the lengthened forward step (seen in the vertical component), the weakened bending forces, the slower braking, and the generally slower movements, etc.

* Biofizika **3**: No. 5, 582–589, 1958.

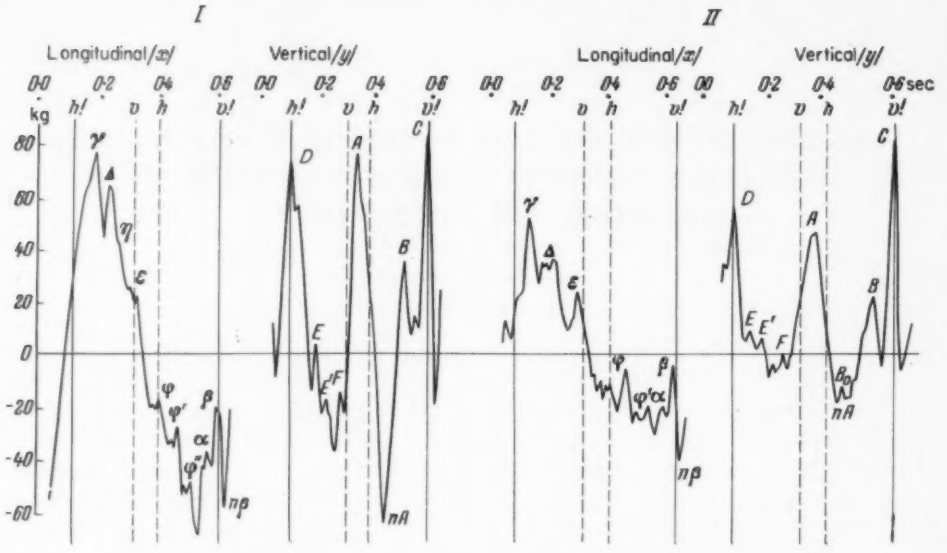

FIG. 1. The force components at the common centre of gravity of the leg in a trained athlete running 400 m (I) at the start and (II) near the finish. Symbols as in Table 2. The vertical lines correspond to the instants of the backward (*h!*) and forward (*v!*) steps (the broken lines relate to the contralateral leg). All the forces fall off towards the finish, except (*C*).

All the other changes listed in Table 1 can be considered in the same way. Only the main phases will be dealt with, though.

(1) *The backward kick.* This action is most important in running [2]; it largely determines the performance over short distances, where high speeds are essential, and is therefore always increased (in its vertical components) in the trained athlete. Fatigue causes its amplitude to decrease, mainly in short runs; in long runs or with untrained runners the reduction is slight. Some increase is also seen if obstacles are overcome or additional (kicking) motions are required.

(2) *Forces at the start of the off-ground period.* These depend very largely on the amplitude of the backward kick, are largely reactive, and are produced by bending the leg at the knee (Δ) and partially by moving the bent leg forward (ϵ). They also change appreciably; fatigue reduces them, though the longer the distance and the better the training the less the fall. The largest changes occur with obstacles (e.g. barriers, when the leg motions are largely determined by reactive forces) and in kicking (where the bending and unbending forces are much increased).

(3) *Forces at the end of the off-ground period* (leg straightening and lowering). Here are found at least two active movements (unbending ζ and braking, nA) and one reactive (α, moving thigh downwards), which is a consequence of the active ones.

Those in the first group are always increased by training (particularly in short-distance running) and are very sensitive to fatigue: they are very important in overcoming obstacles and in kicking motions. The second group is as a whole reactive and accompanies the first; the magnitude of the forward stride as a whole depends on the preceding forces, as Table 1 shows.

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TABLE 1. CHANGES IN THE DYNAMIC COMPONENTS DURING RUNNING

Component	Changes produced by training		Changes caused by fatigue				Changes produced by other causes	
	Short distances	Long distances	In untrained runners	In 400 m runs	In medium runs	In long runs	Overcoming obstacles	Kicking movements
Support period Forward step (in $n\beta$)	Increased	Unchanged	Almost unchanged	Reduced	Reduced	Almost Unchanged	Reduced	Increased
Forward step (in C)	Somewhat increased	Almost unchanged	Slightly increased	Somewhat increased	Increased	Almost unchanged	Increased	Slightly increased
In resultant of forward step	Rotated upwards	Rotated upwards	Almost unchanged	Rotated somewhat upwards	Rotated upwards	Rotated somewhat upwards	Rotated upwards	Almost unchanged
Backward step (in γ)	Slightly increased	Unchanged	Slightly reduced	Reduced	Reduced	Almost unchanged	Increased	Slightly increased
Backward step (in D)	Somewhat increased	Slightly increased	Slightly reduced	Reduced	Reduced	Almost unchanged	Increased	Increased
In resultant of backward step	Rotated upwards	Rotated upwards	Almost unchanged	Rotated upwards	Rotated upwards	Rotated slightly upwards	Rotated upwards	Rotated forwards
Transfer period Use of reactive forces (Δ and E)	Somewhat increased	Somewhat increased	Reduced	Somewhat reduced	Reduced	Slightly reduced	Very greatly increased	Almost not detected
Flexing forces (ϵ)	Unchanged	Unchanged	Somewhat reduced	Unchanged	Slightly reduced	Somewhat reduced	Increased	Greatly increased; straightening forces added
Braking (nA)	Greatly increased	Slightly reduced	Somewhat reduced	Greatly reduced	Slightly reduced	Unchanged	Increased; reactive forces used	Not detected
Reactive force (α)	Appears	Appears	Not found	Increased	Increased	Slightly increased	Increased	Greatly increased

Let us consider the effects of training and fatigue on some of the more important components (main bending forces, forces moving the leg forward when bent at the knee, and those responsible for braking). There are indications that these forces have different central origins, because those of the first type appear in the primitive running of a child, and determine the tempo. These first forces are amongst those few which are always completely under the control of the individual [2], and so must be controlled by the highest centres. The braking is not subject to control at all, and the forces rise and fall in exact sequence with the thigh motion [2]. These movements cause no extension of the rear calf muscle. Bernshtein has supposed that the forces are determined somewhat by myotatic reflexes to extension [2], so that very low-lying centres can control the activity (e.g. ones at the lumbar level of the spinal cord); there is evidence [3] that central control is exerted via these centres.

Our studies indicate that the first forces (Bernshtein's "earliest occurring locomotor impulses") are not greatly influenced by special training in running (see Table 1), while the braking forces become quite large for high-speed running, but show much less of an increase for long-distance runs. The way the force relationships change in fatigue depends on the running speed. The first forces are reduced in slow, long-distance runs, while the braking is not much affected, whereas in 400 m fast runs the first forces are normal but the braking is much reduced (Table 2).

These changes are clearly important to studies of trained running and fatigue in relation to the states of the corresponding nervous centres. We might at first sight suppose that in fast running the fatigue occurs primarily in the co-ordination apparatus; although this is logical, it is not based on physiological facts, since no direct data on the states of the appropriate centres exist. We therefore avoid drawing any conclusions, the more so since this is outside the purpose of the present paper. Extensive studies of the nervous centres, the muscles, etc., would be required to settle the question.

There is no doubt, though, that the forms the fatigue takes in runs of different lengths are different; this is indicated by the energy requirements. Table 2 shows

TABLE 2. ENERGY DEMANDS IN VARIOUS TYPES OF RUNNING

Distance	Maximum speed (m/sec)	Maximum pace (steps/min)	Basic requirements of fresh subjects			Maximum fall in force at finish (%)	
			Total per kilogramme for 1 min in support period	Braking force (kg)	Flexing force (kg)	Braking force (nA)	Flexing force (ϵ)
Trained							
400 m	7·95	235	537·0	−65·4	20·0	70·9	6·4
800 m	7·50	214	340·0	−45·4	27·3	55·6	0·1
5000 m	5·90	175	231·0	−16·1	26·0	18·5	30·1
Untrained							
400 m	5·50	197	230·0	−23·3	24·1	40·6	60·4
1600 m	6·50	200	257·0	−25·1	26·7	57·1	32·5

that the energy consumed varies greatly with distance run; 400 m requires the most, 5000 m the least, with 800 m falling in between. The same conclusion follows from the ratio of the weight to the sum of the forward and backward forces for 1 min, i.e. the energy consumed in accelerating and braking 1 kg of body weight for 1 min. For 400 m the value is 537 kg/kg, for 800 m it is 340 kg/kg, and for 5000 m, 231 kg/kg. The braking force is inversely dependent on the distance, whereas the reverse tends to be true of the other forces (Table 2). This does not apply to untrained runners, where all components fall off identically.

We therefore have some reason to believe that the various types of fatigue may be related to disturbances in the co-ordinating or higher centres. The limiting speeds and high energy demands of short runs produce a kind of fatigue different from that found in slower runs over long distances; this applies only to trained runners, however. The way the force relationships vary and the roles of these co-ordination relations in various types of running can be used in classifying the force aspects of motion.

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SUMMARY

The dynamic components of locomotor acts must be studied to solve co-ordination problems, no matter whether the acts are pure or (to use Orbeli's term) "adjusted" [4]. The data (see [1] for those on walking) can be used to divide the dynamic components up into three groups, in accordance with the role of each force: 1. main forces, 2. accessory forces and 3. secondary or derivative forces.

The data are collected together in Table 3 for convenience in demonstrating how the motions can be specified in terms of the forces.

The main forces. These play the main parts, without which the motion cannot be appropriately performed. They include the following.

(a) In walking on the level, the backward and flexing forces.
(b) In walking up a slope, the backward thrust, assisted by additional leg-straightening forces.
(c) In walking down a slope, the forward step and (possibly) straightening forces.
(d) In fast running, the backward, flexing, and braking forces.
(e) In slow running, the backward, flexing, and (it would seem) straightening forces.
(f) In overcoming obstacles, the backward, straightening, and braking forces.
(g) In kicking movements, the backward and flexing forces.

These forces must always be considered in studies on co-ordination.

The accessory forces. Numerous forces which, while not responsible for the major features, affect many details are assigned to this group. They include the following.

(a) In walking on the level, the straightening and braking forces.
(b) In walking up a slope, the flexing forces and a small additional forward force on the leading leg.
(c) In walking down a slope, the flexing force on the supporting leg and the braking before contact.
(d) In fast running, the braking, straightening, and reactive forces.
(e) In slow running, the braking and reactive forces.

TABLE 3. THE DYNAMIC COMPONENTS AND CO-ORDINATION

Component	Walking			Running		Overcoming barrier	Kicking
	On level	On a rise	On a descent	Fast	Slow		
Support period							
Forward step	Derivative	Almost absent	Main	Derivative	Derivative	Derivative	Derivative
Backward step	Main	Main	Almost absent	Main	Main	Main	Main
Additional forces	—	Lift of supporting leg	Lowering supporting leg	—	—	—	—
Transfer period							
Flexing forces	Main	Accessory	Accessory	Main	Main	Accessory	Accessory
Use of Reactive forces	—	—	—	Accessory	Accessory	Accessory	—
Straightening forces	Accessory	Almost absent	(Main)	Accessory	Accessory	Main	Main
Braking	—	—	—	Main	Accessory	Main	Accessory
Reactive forces	Derivative	—	Derivative	Derivative	Derivative	Accessory	Derivative
Braking before support period	Accessory	—	Accessory	Accessory	Accessory	Accessory	Accessory
Accessory forces	—	Additional upward lift	—	—	—	Extensive use of reactive forces	Extra flexion and straightening

(f) In overcoming obstacles, the flexing and the reactive forces resulting from braking and other motions.

(g) In kicking, the flexing and braking forces.

These forces must always be studied to give a complete picture of how the movement is done.

Derivative forces. These are forces produced by reaction from other limbs or on contact with the ground. All peaks in the vertical components during walking belong in this group (i.e. for the ipselateral and contralateral legs; this applies to running also), as do the reactive parts of the longitudinal components acting at the centre of gravity of the thigh (α); these are found in walking and in running [2]. They are inevitable, but must not be neglected, since they, too, determine the performance.

This classification should not be taken as final and definitive, or as a dogma. It will be supplemented or altered by further work, but can be used to establish the main force components in locomotor acts, and is of great value in analysis.

Translated by J. E. S. Bradley

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METHODS AND APPARATUS

AUTORADIOGRAPHY OF FROZEN TISSUES*

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Freezing of soft tissues to obtain autoradiographs of whole sections has been employed in several works [1–3]. Pellerin (3), when devising his method, had in view the prevention of artefacts produced by the diffusion of substances after death and by the chemical action of tissue on photographic emulsion.

The method which has been devised by us independently of Pellerin has a number of distinctive features. In addition, it is impossible to agree with Pellerin's statement that the sensitivity of photographic emulsion is unaltered on decreasing the temperature of exposure, a matter which we shall consider in detail below.

Many authors using autoradiography direct their attention mainly to the end result,

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i.e. the blackening of the photographic emulsion, attempting to apply to it one or other physiological interpretation. As regards the methods of preparatory treatment of tissues for autoradiography, this side of the matter is often left in the dark and not subjected to sufficiently critical appraisal. In the meantime, the facts available allow it to be stated that the preliminary treatment of the tissues and the conditions of their exposure on photographic emulsion predetermine the picture which is obtained of the localization of the labelled substances during life in the biological material under study.

Leaving aside secondary and easily rectified causes of distortions we shall examine only the factors which are of principal significance in the physiological interpretation of autoradiographs.

The chemical action of tissues on photographic emulsion

It has been comprehensively demonstrated [4, 5] that the chemical action of tissues on photographic emulsion is comparable in its activity with the action of the indicator doses of the radiaoctive isotopes. With this in view, in autoradiographic procedure attention was first of all turned to a search for means of neutralizing this factor as a main source of artefact production. The use of water-impermeable layers between the object and the photographic emulsion proved to be effective. However, reduction in resolution, which to a marked extent is dependent on the size of the gap between object and photographic emulsion, is a shortcoming of such a procedure. Moreover, layers of a thickness greater than 15–18 mμ exclude the possibility of using such isotopes as ^{14}C, ^{35}S and ^{45}Ca as radioactive indicators. To avoid this, resort is often made to careful drying of the preparations to complete dehydration; the preparations are then covered by a waterproof film of a thickness of the order of 1 mμ or they are placed in direct contact with the photographic emulsion and exposed at a temperature around 0°C in hermetically sealed glass or metal vessels containing a water absorbing agent.

Autoradiographs obtained by such methods are free of artefacts arising from chemical action of the tissue on photographic emulsion, but they cannot yet be regarded as documents which reflect the actual localization of the radioisotope during life in the material being studied. They incorporate artefacts produced by the diffusion of substances after death.

Diffusion of substances after death as a source of artefacts in autoradiography

The effect of diffusion of substances cannot always be detected in autoradiographs of relatively large objects. However, the existence of this factor is readily apparent in fine details, for example in the hairs found on the surface and along the edges of the leaves of certain plants. In support of this we shall cite the results of our experiments on geranium leaves. Autoradiographs of leaves of this plant which contain the radioactive isotope of sulphur ^{35}S are shown in Fig. 1. Preparation of the first leaf involved drying in a desiccator (Fig. 1*a*), the second received no such treatment (Fig. 1*b*). Both leaves were placed in an X-ray film cassette on unscreened film. The cassette was then lowered into a Dewar flask of liquid nitrogen, in which the leaves were exposed for 120 hr.

From comparison of the two autoradiograph illustrations it can be seen that the hairs of the dried leaf contained the isotope, while the isotope is absent from the hairs of the freshly prepared leaf. The reason for this, it seems, is that diffusion of substances occurred in the time which elapsed between cessation of cellular metabolism

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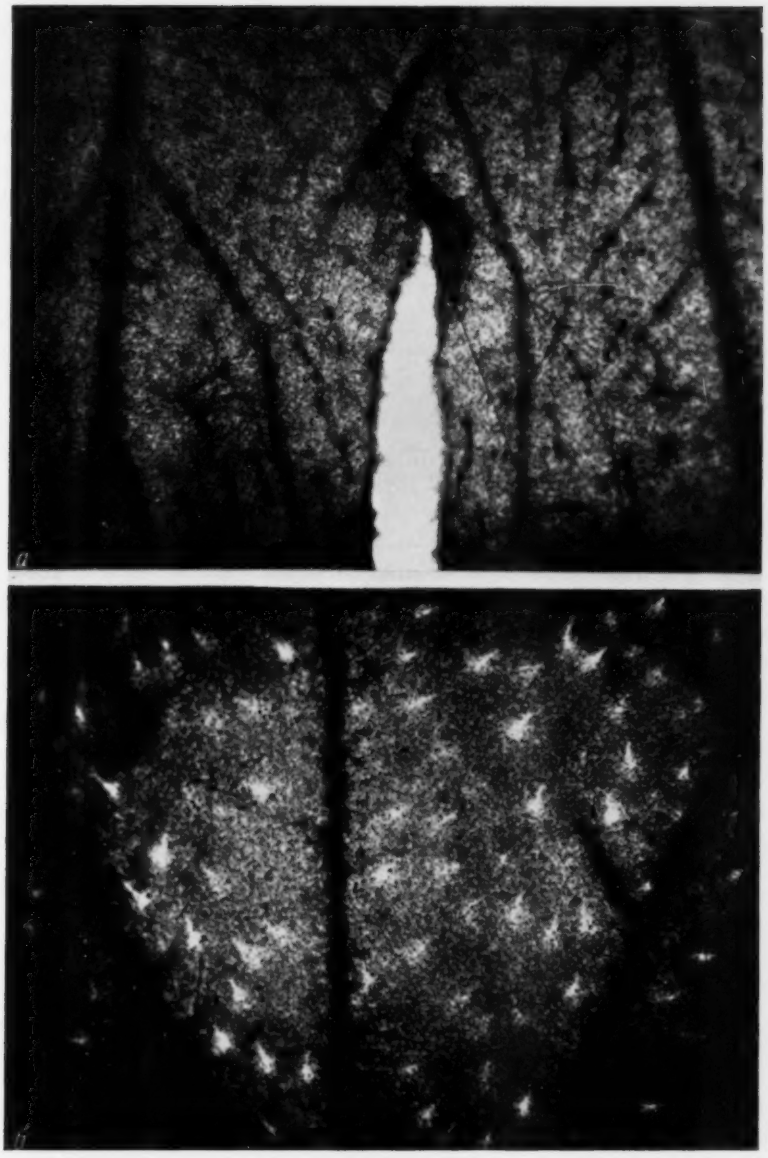

FIG. 1. Autoradiographs of geranium leaves. (*a*) Autoradiograph of a dehydrated leaf. The hairs on it have the appearance of diffuse black points and lines. (*b*) Autoradiograph of a freshly prepared leaf. The white sharp-pointed sectors of emulsion are the shadow images of the non-radioactive hairs.

during the drying process and final dehydration. On plunging the fresh leaf into liquid nitrogen death of cells occurred simultaneously with fixation of substances by the low temperature, and the maintenance of the leaf in low temperature conditions during its exposure on photographic emulsion excluded also the possibility of any chemical alterations in the cells.

The sharpness of the image of the hairs of a leaf depended on the emission energy of the isotope used. It was greater the shorter the path of the β-particles in tissue. The length of path of ^{35}S β-particles in tissue is about 20 mμ, i.e. approximately one third of the average thickness of the hairs of the geranium leaf. On account of this the hairs prevented penetration of β-particles to the portions of the photographic emulsion occupied by the hairs and they became visible in the autoradiograph (the β-particles originate in the leaf-tissue above the hairs).

It must be concluded from the experiments done, that in use of the autoradiographic technique, together with artefacts resulting from the chemical action of tissues on photographic emulsion, artefacts are observed which arise by diffusion of substances after death.

Fixation of tissues by freezing

Besides the enumerated methods of preparing tissues for autoradiography, tissues are often fixed using various liquid solutions. However, it is well known that the solutions employed in histology contain water, alcohol and other solvents which remove from the tissue ions, water-soluble substances and fat-soluble components. Apparently no fixatives exist which would not introduce more or less deep-seated chemical and physical alterations in tissue.

Fixation by freezing is basically distinct from all the common procedures for preparing tissues for autoradiography.

(1) It instantaneously stops the metabolic activity of an organism and completely prevents diffusion and chemical interaction of substances in the frozen tissue.

(2) Frozen objects allow various methods of preparation to be employed without disturbing the disposition of individual organs, and of liquid and semi-liquid masses.

(3) Frozen tissue can remain in contact with photographic emulsion for a long time at low temperature without the formation of any chemical artefacts whatsoever.

In the normal event the intracellular structure of a tissue is destroyed by the ice crystals which form in it during freezing. However, by observing certain conditions guaranteeing a sufficiently high rate of freezing, it is possible to fix pieces of tissue about 1 mm thick without harming the intracellular structure, from which histological sections can subsequently be made to study the intracellular localization of radioactive isotopes.*

The intactness of the intracellular structure does not have a substantial role to play in macro- and semi-microscopic studies of the distribution of radioactive indicators.

The technique of freezing small animals and making whole sections

Mice, which had received the isotope ^{45}Ca in the form of a water-soluble solution of $^{45}CaCl_2$ or ^{35}S-labelled vitamin B_1, were the experimental materials used. Freezing

* The reader can find more detailed information in another paper by us [6].

was carried out 1 hr after injection of the isotope. An animal was narcotized with ether for convenience in fastening it to a flat wooden holder. A thick layer of heavy casein glue was spread on the holder and the animal was firmly pressed into it. The glue, filling the free space and gripping the limbs and part of the head, firmly fixes the animal on to the holder. Gradual lowering of the holder and animal into liquid nitrogen avoids cracking and loosening of the glue. A mouse of 25 g reaches the temperature of liquid nitrogen in 1·5–2 min.

We work on the principle that such a rate of freezing small intact live animals ensures a rapid cessation of the life processes of the cells and creates conditions where diffusion of substances after death is impossible.

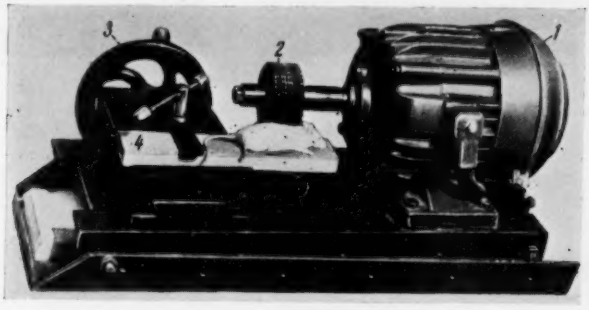

FIG. 2. A device for making whole sections of small animals. (1) electric motor; (2) block of circular saws; (3) flywheel with handle for transverse movement of the support; (4) a holder in the form of a wooden strip 60 × 220 × 20 mm in size for fixing the animal.

The frozen animal can be sliced longitudinally or transversly by means of a chilled block of circular saws. We used the arrangement, shown in Fig. 2, to obtain 2–3 mm thick sections. The heat capacity of the system is such that thawing of the object, which would affect the distribution of substances, does not occur during rapid sectioning. Sections of other animals and plant materials can be prepared in like manner.

The procedure for obtaining autoradiographs of frozen materials

Transverse sections of a mouse prepared by the technique described above were placed on the bottom of an X-ray film cassette which had first been cooled in liquid nitrogen (the cassette and a piece of unscreened X-ray film inserted into it were cooled simultaneously). Exposure was made in a container of dry ice at −72°C.

Autoradiographs obtained in this way are shown in Figs. 3 and 4. Some other apparatus is required in working with fine material, such as haricot seed, wheat grains, etc. A tissue holder in the form of a brass face plate was prepared for this purpose (Fig. 5). A depression in it was as before filled with thick casein glue, into which was pressed the material ready for autoradiography. The tissue holder was then transferred by means of long tongs into a Dewar flask containing liquid nitrogen for freezing. After the block had reached the temperature of liquid nitrogen the shank of the face plate was inserted into the spindle of a bench lathe and the block was cut to produce a flat surface with a previously chilled blade (Fig. 6). With a block of

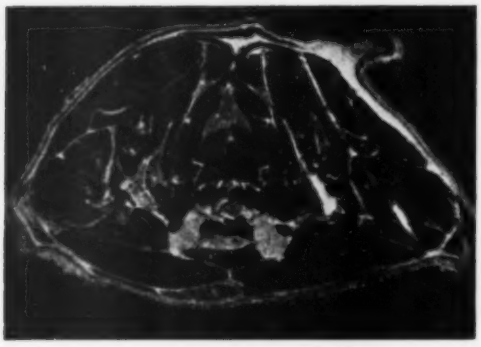

FIG. 3. Positive print of an autoradiograph of a transverse section of a mouse's head containing ^{45}Ca. The isotope was injected intraperitoneally as a 1 ml solution of $^{45}CaCl_2$ at a dose of $4\mu c/g$ weight; the section is through the brain region. In the autoradiograph it can be seen that ^{45}Ca is localized mainly in bones.

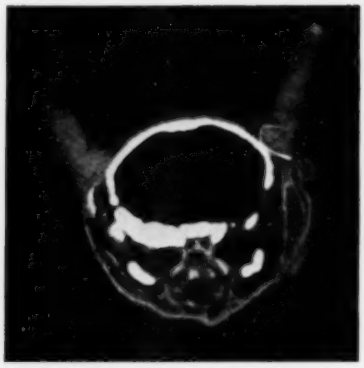

FIG. 4. Autoradiograph of a transverse section of a mouse made by injection of ^{35}S-labelled vitamin B_1 through the caudal vein. All other conditions are as described under Fig. 3. In the autoradiograph it can be seen that vitamin B_1, like ^{45}Ca, does not enter the brain or the cranium. It is concentrated mainly in membranes and skin.

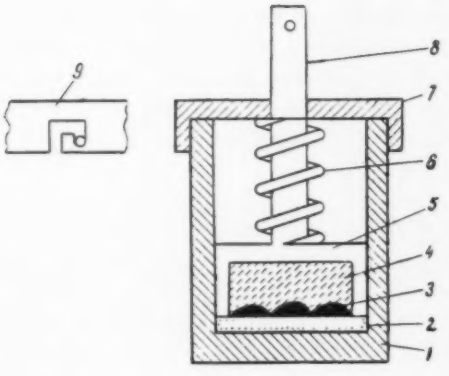

FIG. 5. The cassette for exposing frozen objects in liquid nitrogen or in a container of solid CO_2. (1) body of the cassette; (2) photographic film; (3) experimental materials; (4) casein glue; (5) face plate; (6) compressed steel spring; (7) lid; (8) shank of face plate; (9) arrangement for locking on cassette lid. The cassette (apart from the spring) is made out of brass.

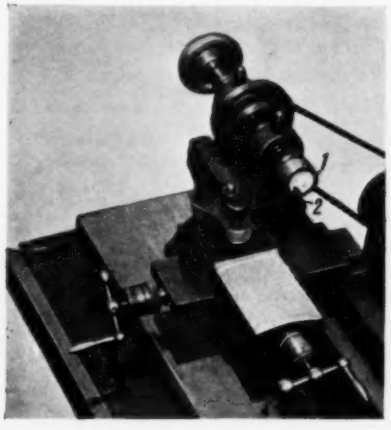

FIG. 6. The bench lathe arrangement for working the block surface of the frozen materials in the tissue holder. (1) the tissue holder, the shank of which is fixed on the spindle; (2) the cutting tool, a round-edged spade-shaped instrument inclined to the left; (3) and (4) hand levers for providing transverse and longitudinal motion of the support.

sufficient size this operation could be carried out in one step without supplementary cooling. On completion of trimming on the lathe the block was again transferred to liquid nitrogen. A cassette along with a piece of X-ray film cut in the shape of a circular disc was also cooled in liquid nitrogen. To prevent warping of the film it was necessary to flatten it on the bottom of the cassette during the cooling process with an identical face plate without a depression. After carrying out these operations the cassette was assembled and transferred for exposure to a container of dry ice or to a flask of liquid nitrogen.

Autoradiographs of transverse sections of haricot and wheat seed are shown in Fig. 7. This technique can be used for autoradiographic study (in various sectionings) of insects, worms, and other small objects. In particular it has been employed in studying the uptake of tritium water during immersion of seeds. In this case freezing and exposure of the frozen seed was carried out in liquid nitrogen. As an example, autoradiographs of transverse sections of haricot seeds, produced by permeation into them of tritium, are shown in Fig. 8.

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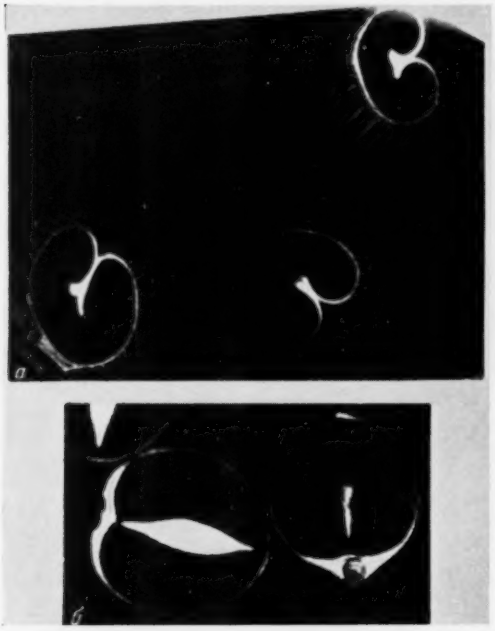

FIG. 7. Autoradiographs of transverse sections of wheat and haricot seed soaked in an aqueous solution of Na_2SO_4 labelled with ^{35}S. (a) Practically no ^{35}S has entered the wheat seed, and it is concentrated in the integument; (b) section of haricot seed, where the sulphur is also concentrated in the external integument of the seed and penetrates into the intralobular space. The picture is somewhat more complicated in the embryo.

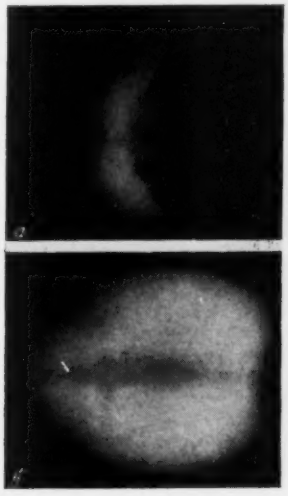

FIG. 8. Autoradiographs of transverse sections of haricot seed soaked in tritium water. In the autoradiograph it can be seen that tritium water is not held in the integument but penetrates inside the seed in relation to the length of the period of soaking. (*a*) 8 hr immersion, (*b*) 12 hr immersion.

The exposure of frozen tissues on photographic emulsion

The making of autoradiographs of frozen biological materials is bound up with the necessity of providing conditions under which thawing shall not occur. The major portion of a biological tissue, for example frog gastrocnemius muscle, freezes at a temperature around $-10°$. But individual components and salt systems, it seems, have a considerably lower freezing point. Therefore it is desirable to expose frozen tissue at a lower temperature than the freezing point of the tissue, for example, at the temperature of dry ice ($-72°$). This substance is the most reasonable and easily available for the freezing and exposure on photographic emulsion of the majority of biological materials. The exposure of frozen tissues is in addition bound up with a reduction in sensitivity of photographic emulsion.

The influence of temperature on the sensitivity of photographic emulsion

While obtaining autoradiographs of frozen tissues it became evident that the sensitivity of photographic emulsion declined on lowering the temperature of exposure. An X-ray photographic emulsion of Soviet origin was tested in a more detailed investigation of this question. The relationship observed is presented in Fig. 9; the temperature is marked along the abscissa, and the exposure time along the ordinate. The unit of exposure time is taken as that during which a sample, containing ^{35}S, blackens the emulsion to an optical density of $D = 1{\cdot}4$ at a room temperature of approximately $+20°$. It can be seen from the graph that the exposure time at the temperature of solid CO_2 ($-72°C$) is approximately 2·5 times greater, and at the temperature of liquid nitrogen $-(193°C)$ approximately 5 times greater than that required to produce the same blackening at room temperature.

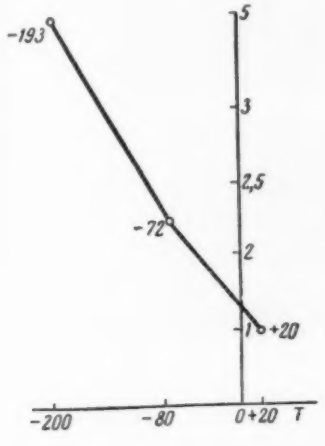

Fig. 9. The relation of exposure time to temperature.

Such a result is in agreement with the generally accepted theory of latent image formation of Gurney and Mott [7]. According to this theory, the final part of the process is characterized by electrolytic transfer of silver ions to electron traps where, being neutralized, they form latent image particles. The mobility of ions, as is well known, is dependent to a marked extent on temperature. At −193°C, it seems, only a small number of silver ions can be neutralized. This process can, in part, be brought to a conclusion by warming the emulsion from −193°C to the temperature of development. The very great decline in sensitivity of photographic emulsion at low temperatures can be explained in this way.

In one investigation [8] ordinary optical emulsion was exposed to visible light at temperatures from + 58 to − 193°C. The results of these studies showed that, at a temperature around −72°C the sensitivity of ordinary photographic emulsion is reduced by slightly more than twice, and at −193°C the sensitivity is insignificant. Dollmann [9] studied nuclear emulsions 50 and 100 mμ thick, irradiating them with neutrons of an energy of 10 MeV over a temperature range from +20 to −66°C, and it was demonstrated that the sensitivity of nuclear emulsion at −66°C is reduced approximately three times. The material quoted convincingly shows that the reduction in sensitivity of photographic emulsion with decrease in temperature of exposure is a property inherent in it and, apparently, depends only to a very small extent on the type of emulsion. All the facts which have been stated do not permit us to agree with Pellerin's assertion that the sensitivity of photographic emulsion is not altered by lowering the temperature of exposure.

On the basis of what has been stated above it can be concluded that the use of frozen tissues opens up a series of new possibilities in the further development and perfection of the technique of studying biological processes by autoradiography.

The results presented in this paper, we believe, will assist the application of this promising technique in scientific research.

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SUMMARY

(1) The problem of artefact formation during preparation and exposure of biological materials on photographic emulsion at room temperature has been examined. It has been shown that, during dehydration of material for autoradiography, artefacts arise as the result of diffusion of substances after death.

(2) A procedure for obtaining autoradiographs of frozen tissues at low temperature is described. It has been shown that the sensitivity of photographic emulsion is to a marked extent dependent on the temperature of exposure.

Translated by C. R. PRINGLE

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THE DIFFERENCES OF PRINCIPLE BETWEEN BALLISTOCARDIOGRAPHY AND DYNAMOCARDIOGRAPHY*

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Ballistocardiography, which was developed by Starr *et al.* [1], is widely used in cardiac studies; another technique (dynamocardiography†) has been developed for the purpose in the U.S.S.R. It is of interest to compare the physical principles of the two methods, and the curves that they give.

The mechanical effects of cardiac activity can be divided into two groups, of which the first consists of effects in the cardiovascular system itself. These effects are made up of cardiac kinematics (change in shape, size and position of the heart) and of the dynamics of blood flow (mass displacements, pressure waves, etc.); they all produce responses in the body, considered as an inert mass. These responses make up the second group of effects, which include displacement of the body relative to a fixed surface during each contraction, and changes in the mass relationships of the various parts of the body. These changes cause the effective centre of gravity of any part of the body to move during the heart cycle.

Methods of studying the first group of effects are classical (cardiography, sphygmography, intravascular and intracardiac pressure measurement, etc.); the second group can be studied by ballistocardiography and dynamocardiography; in the first method body displacements are recorded, in the second displacements of the partial centre of gravity.

(1) THE DIFFERENCES BETWEEN THE PHYSICAL PRINCIPLES OF BALLISTOCARDIOGRAPHY AND DYNAMOCARDIOGRAPHY

As long ago as 1877 Gordon [3] developed a method of recording body displacements; many highly developed ballistocardiographs are now available. The platform on which the body rests is displaced because the body reacts on it; the displacements are recorded as a ballistocardiogram curve. The amplitude A_b of this curve is proportional to the applied force F:

$$A_b = kF \tag{1}$$

where A is a sensitivity coefficient. The displacement in general occurs in an arbitrary

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† The original name given to it was cardiohaemodynamography [2].

direction in space [4], so in the general case the platform will be acted on by forces F_x, F_y and F_z, as shown in the diagram (Fig. 1).

It is more difficult to record displacements of the centre of gravity (dynamocardiography); torque detectors are used. Analysis [5] of torque detector response shows that the amplitude A_d is defined by

$$A_d = (k'/L)Pl, \tag{2}$$

where P is the modulus of the vertical forces, l is the distance of these forces from the neutral line of the torque transducer, L is half the length of the transducer and k' is a coefficient of proportionality.

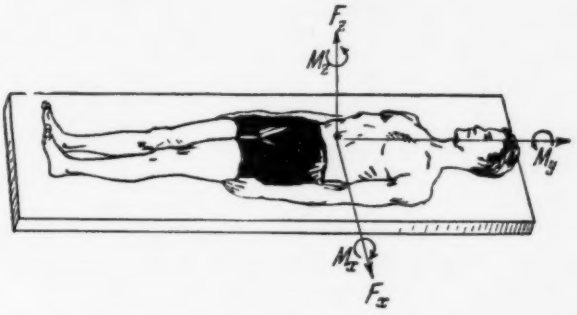

FIG. 1. Schematic representation of the forces (F) recorded in ballistocardiograms, and of the torques (M) recorded in dynamocardiograms.

It is clear from (1) and (2) that the two methods differ radically in principle and hence record different aspects of cardiac activity. It is clear from (2) that the dynamocardiograph can record the displacements normally recorded by the ballistocardiograph, since horizontal displacements will alter l and vertical ones P. Let us consider how F_z (dorsoventral ballistocardiogram) affects the dynamocardiogram. The dorsoventral ballistocardiogram is much weaker than the longitudinal and lateral ones [6]. Taking an average of F_z, and assuming it applied to the end of the transducer (vertical forces maximally recorded, i.e. $l/L = 1$), we get a torque less than that commonly recorded in dynamocardiography. The torque is still less if F_z is applied in such a way that $l/L < 1$; F_z produces no torque at all if $l = 0$. The argument is, of course, equally valid if F_z is applied to the other side of the neutral line (l negative).

Hence F_z is not recorded in any definite fashion in the dynamocardiogram. The reason for this is that the components that go to make up F_z act at various points and have various magnitudes; the overall effect is small, because the components on the two sides of the neutral line tend to cancel out. We can therefore neglect the effect of the dorsoventral ballistocardiogram on the dynamocardiogram as being small, as a comparison of the two curves in Fig. 2 shows. The two curves show no similarities at all.

We next consider how the horizontal displacements affect the dynamocardiogram, in relation to the longitudinal ballistocardiogram (F_y). In principle there are two effects; the first is that the heart and major vessels (the sources of the forces) lie 10–15 cm

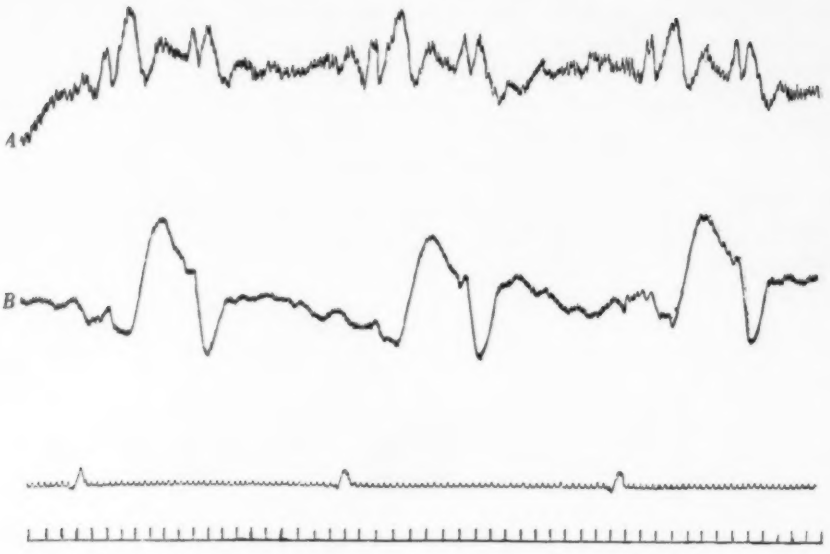

FIG. 2. Dorsoventral ballistocardiogram (A) and dynamocardiogram (B) recorded in synchronism.

above the platform, and if the heart, etc., were rigidly coupled to the platform this distance could be considered as the radius at which the force producing the torque acts. The torque would then be recorded on the dynamocardiogram. In practice no such rigid link exists, and the actual effect is unimportant, as the following tests show.

A wooden parallelopiped of base area corresponding to the size of the transducer was mounted on the platform; the height h of the block was either 8 or 20 cm. Boxes were placed in the platform some distance away from the block, as shown in Fig. 3, so that the subject was supported on a level surface. Another transducer was placed between the block and the subject, as shown. The records from the two transducers were made synchronously on the same strip. The block acted as a lever, to whose end the force F_y is applied; this produced the torque M_x of Fig. 1, which was recorded. In the first set of tests we used $h = 8$ cm; the two curves (Fig. 4) showed no essential differences and were practically the same as normally found (i.e. with the upper transducer alone). This indicates that the torque produced was small; this point was checked in the second set of tests, where h was made 20 cm. The torque produced

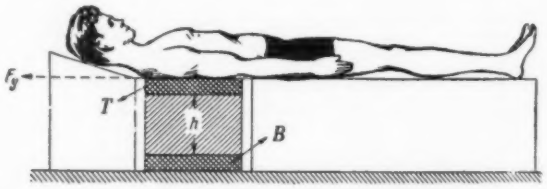

FIG. 3. Effects of horizontal forces (F_y) on the dynamocardiogram. Position of subject used for test. T and B are the top and bottom transducers respectively.

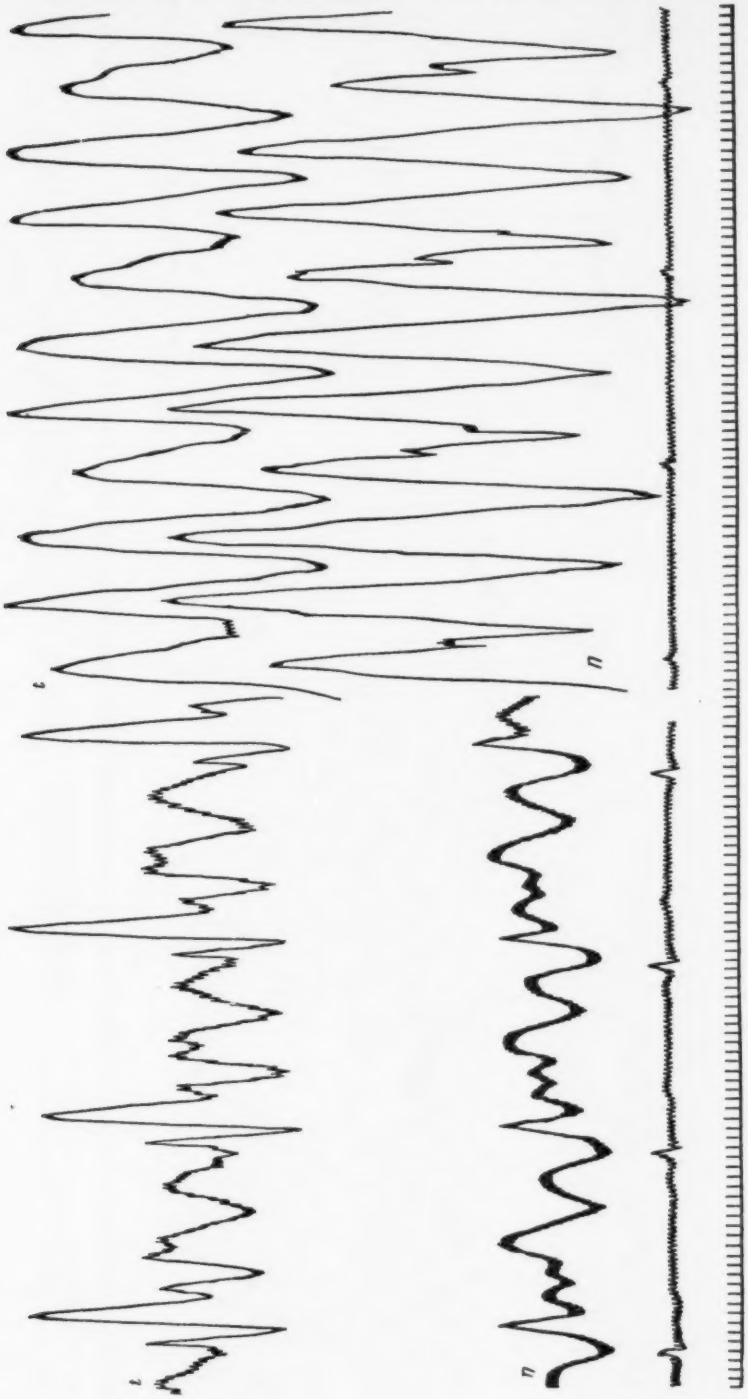

FIG. 4. Effects of horizontal forces (F_y) on the dynamocardiogram. Left-hand curves, $h = 8$ cm; right-hand curves, $h = 20$ cm. The upper and lower curves were recorded with transducers T and B of Fig. 3 respectively. Explanation in text.

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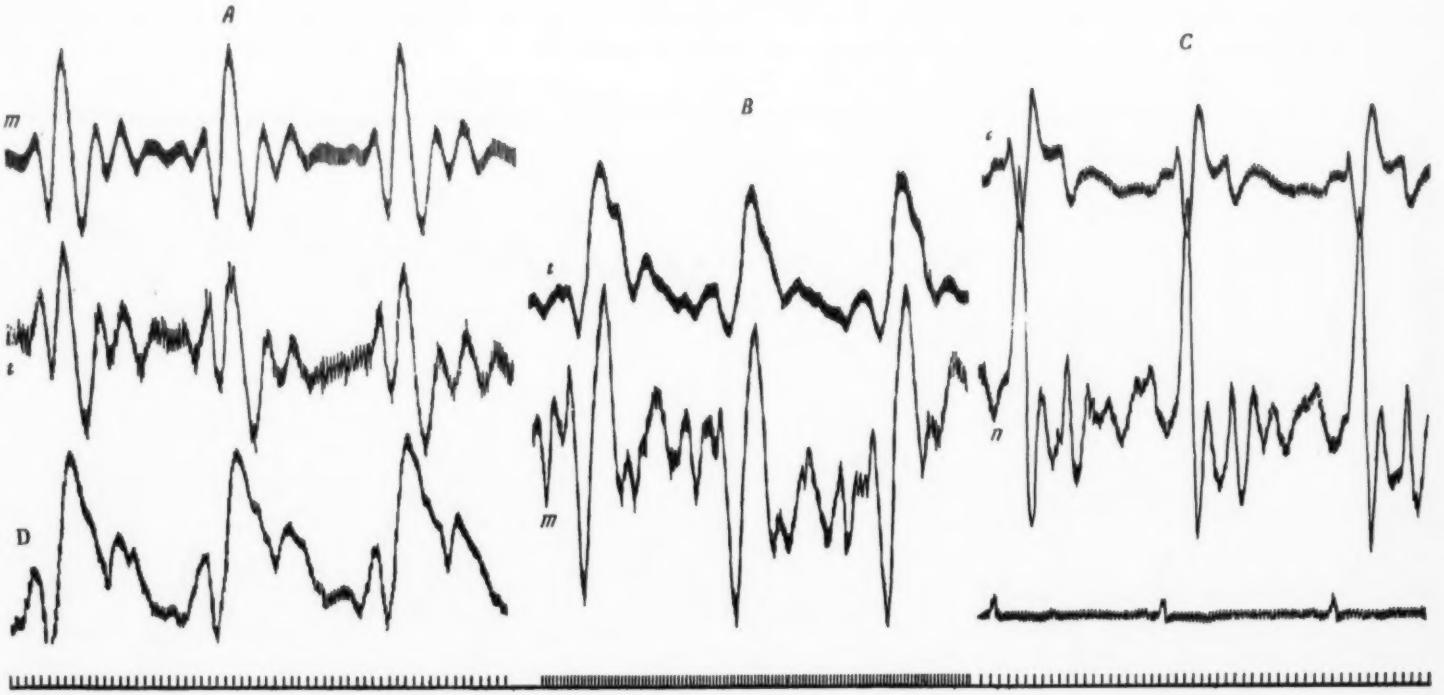

FIG. 5. (*A*) Synchronous record of whole-body (*m*) and thoracic (*t*) ballistocardiograms; *D* = dynamocardiogram. (*B*) Synchronous record of whole-body (*m*) and thoracic (*t*) dynamocardiograms. (*C*) Synchronous record of thoracic (*t*) and lumbar-sacral (*n*) dynamocardiograms. Explanation in text.

by F_y was then increased by a factor of 2·5. Figure 4 shows that the two transducers then gave very different curves, with the one from the lower transducer differing greatly from normal. The dynamocardiograph therefore records F_y only if its torque is greatly increased by artificial means. Hence F_y does not normally affect the dynamocardiogram in this way.

The second way in which the horizontal displacements may affect the dynamocardiogram is as follows. The weight P of the thoracic area (which acts through the centre of gravity) is changed in point of application by the displacements, so l in (2) will vary in the same way as the displacement; the dynamocardiogram will record this. In practice, no appreciable effect of the longitudinal ballistocardiogram on the dynamocardiogram is found, however; the ballistocardiogram curves given by the thorax, leg, head, etc., resemble that from the body as a whole (Fig. 5*A*) [7], whereas the dynamocardiogram curves from these areas show little in common. Figs. 5(*B*) and 5(*C*) illustrate this.

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The ballistocardiograms given by the various areas are identical because the body is displaced as a whole; the differences found in the dynamocardiogram curves have physiological, and not physical, causes. The thoracic and pelvic dynamocardiograms differ because the blood is displaced in different ways in these areas [8]. The movement of blood in the ascending aorta displaces the centre of gravity of the thorax towards the head (dynamocardiogram displaced upwards), whereas that in the pelvic artery moves it towards the legs (dynamocardiogram displaced downwards). If the dynamocardiograph recorded horizontal displacements the two areas of the body would show identical curves. Tests with the wooden blocks show this (Fig. 6). The

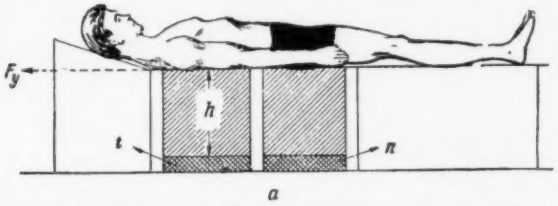

FIG. 6. Dynamocardiograph curves taken with various values of h. Position of subject used for test. See explanation in text and Fig. 7.

torque produced by F_y was made sufficiently large for the dynamocardiograph to record; it was found that the curves recorded in the thoracic and pelvic areas were similar to the ballistocardiogram and had identical directions (Fig. 7).

We therefore conclude that the ballistocardiograph and dynamocardiograph record different aspects of the mechanical effects produced by the heart, and that each method has its place in cardiology.

(2) THE TIME RELATIONS BETWEEN THE BALLISTOCARDIOGRAM AND DYNAMOCARDIOGRAM

The various clinical applications of the two methods arise from their differing physical principles; dynamocardiography can give a detailed phase analysis of the heart cycle [9, 10], whereas ballistocardiography cannot, but is most effective in elucidating coronary circulation disturbances [11, 12]. Dynamocardiography is the

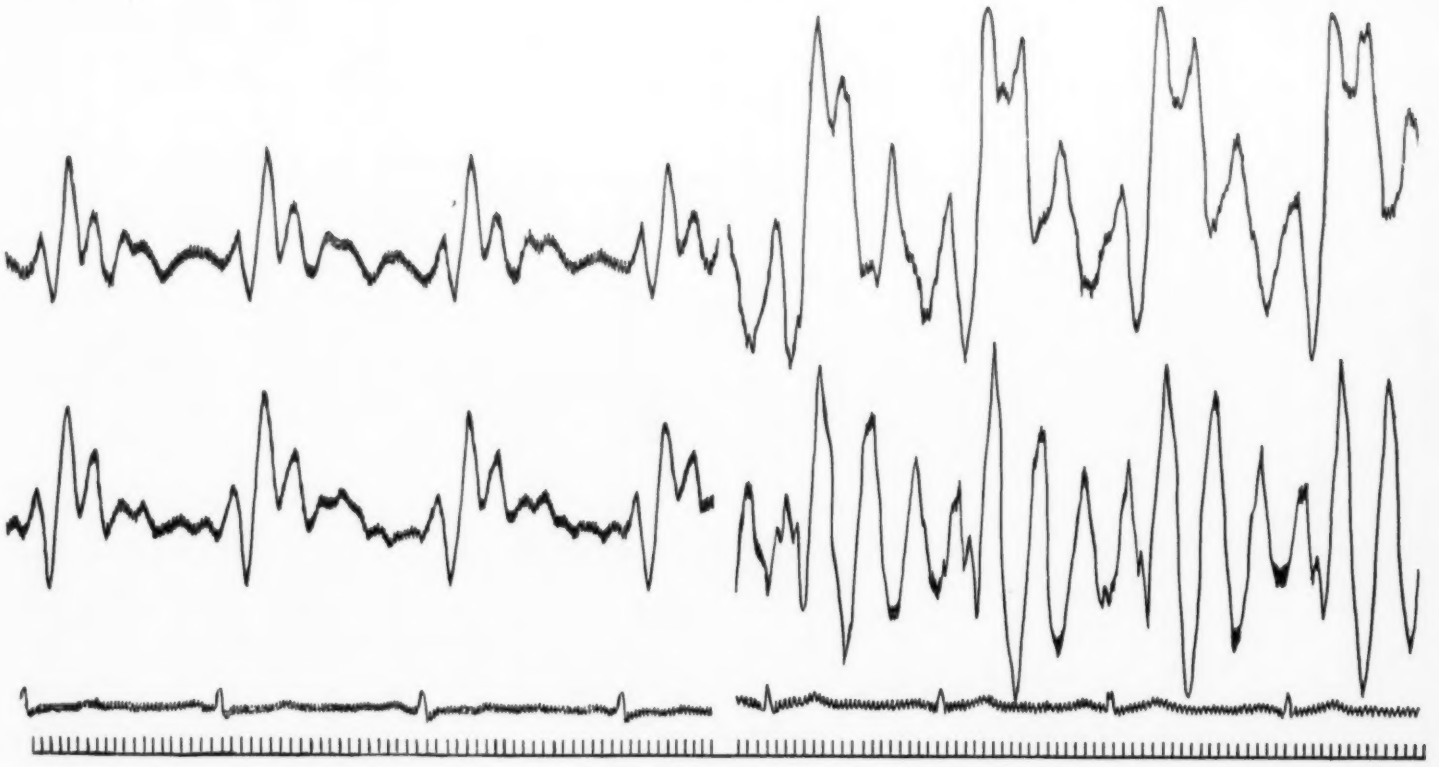

FIG. 7. Dynamocardiograph curves taken with various values of h from the thoracic (t) and lumbar (n) regions. Left-hand curves, $h = 8$ cm; right-hand curves, $h = 20$ cm. Explanation in text.

more useful in vitium cordis [13, 14]; the two methods are therefore not interchangeable, but their appropriate combination gives the fullest information on haemodynamic and contractile disorders [15].

TABLE 1. TIME RELATIONSHIPS OF CHARACTERISTIC POINTS ON BALLISTOCARDIOGRAMS AND DYNAMOCARDIOGRAMS
(Mean values, in seconds)

Ballisto-cardiogram point	Dynamocardiogram points					
	2	3	4	5	6	7
H	+0·046	−0·025	−0·147	−0·234	−0·270	−0·355
I	+0·106	+0·035	−0·087	−0·174	−0·210	−0·295
J	+0·184	+0·113	−0·009	−0·096	−0·132	−0·217
K	+0·276	+0·205	+0·083	−0·004	−0·040	−0·125
L	+0·351	+0·280	+0·158	+0·071	−0·039	−0·050
M	+0·426	+0·355	+0·233	+0·146	+0·110	+0·025

Note. + indicates that the point comes early, − indicates that it comes later.

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It is of interest here to establish the normal time relations between the two types of curve. Longitudinal ballisto- and dynamo-cardiograms were recorded synchronously from 20 healthy individuals aged 25 to 35 years. A multicomponent force-recording apparatus constructed by Stolyarov and Grachev at the Balance and Apparatus Research Institute was used to record all the outputs. The natural frequency of the loaded platform plus man was 40 c/s.

Figure 8 compares the systolic and initial diastolic peaks for waves *G* to *N* on the ballistocardiogram. Point 2 corresponds to the mitral valves opening; it always precedes *H* by about 0·02–0·07 sec, while *H* always precedes point 3. Point 3 itself corresponds to the semilunar valves opening, with the blood beginning to escape from the ventricles; it precedes *I* by 0·01–0·06 sec. *I* frequently corresponds exactly to a notch between 3 and 4 in healthy individuals.

The physiological significance of point 4 is not yet clear; it is supposed that some of the blood ejected from the ventricle has by this time reached the aortal arch. In seven cases 4 coincided with *J*, while in the rest *J* preceded 4 by 0·01–0·04 sec. Point 5 corresponds to the last of the blood being squeezed from the ventricle; in six cases it coincided exactly with *K*, in three *K* was delayed, while in the rest *K* was in advance of 5.

L was always 0·01–0·07 sec behind point 6, which corresponds to the semilunar valves closing, and hence to the start of isometric relaxation of the ventricle. Point 7 corresponds to the end of this phase, when the mitral valves open and the ventricles begin to fill with blood; it shows no definite relation to *M*, and can precede it by up to 0·04 sec, or follow it by up to 0·02 sec. The data given show that *J* and *K* are closely related in time to their corresponding points on the dynamocardiogram; the rest show no exact correspondence in time. This again shows that the two methods relate to physiologically distinct processes. The lack of exact time relationships between the two sets of curves shows that it is useless to try to analyse the phases of the heart cycle from ballistocardiograms.

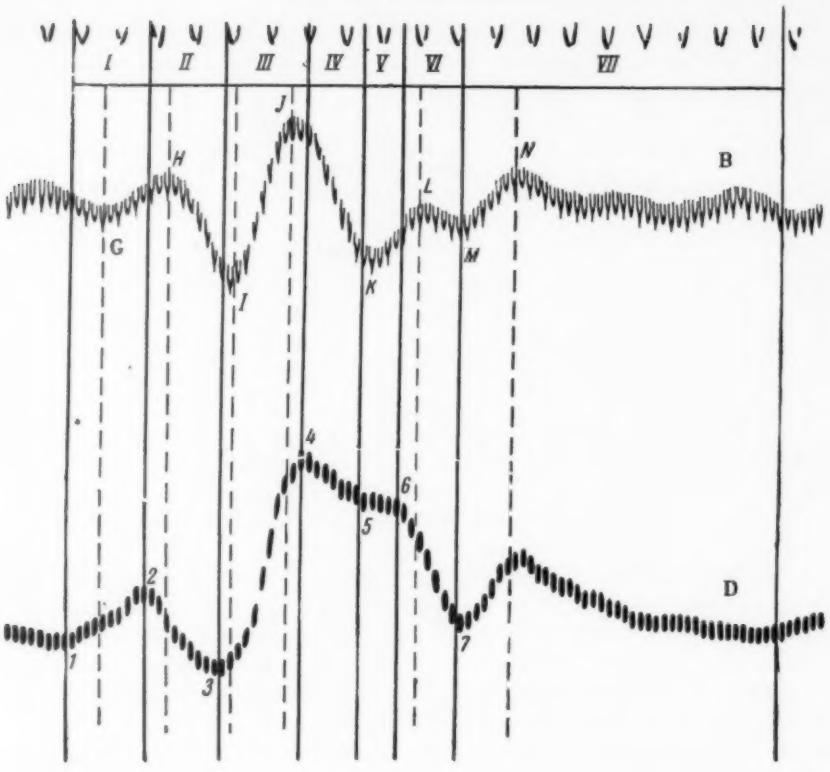

Fig. 8. Synchronous records of the longitudinal displacement ballistocardiogram (*B*) and of the longitudinal dynamocardiogram (*D*). Chart speed 125 mm/sec. The vertical full lines correspond to intervals on the dynamocardiogram related to phases of the heart cycle. (I) auricular systole, (II) isometric contraction phase, (III) and (IV) ventricular expulsion phase, (V) protodiastolic phase, (VI) isometric relaxation phase, (VII) ventricular filling phase. The dashed lines are ballistocardiogram intervals. The time marks on the curves are 0·01 sec apart.

It is now clear why it is physiologically meaningless to term the interval *H* to *K* the haemodynamic systole, as some [16] do. The start, *H*, corresponds to the middle of the distended phase, while the end, *K*, does not always coincide with the end of the flow of blood from the ventricles (point 5). The interval *H* to *K* is therefore always shorter than the true systole, while always longer than the expulsion phase. Its average length is 0·234 sec, while the two phases last 0·292 and 0·218 sec respectively (averages from our data).

SUMMARY

(1) Dynamocardiograms and ballistocardiograms record processes of differing physical natures. The first records the movement of the centre of gravity of the thorax, while the second records displacement of the whole body.

(2) The two sets of processes do not influence one another under normal recording

conditions. The vertical forces recorded on dorsoventral ballistocardiograms from horizontal subjects do not appear on the dynamocardiograms; nor do the horizontal forces recorded on longitudinal and lateral ballistocardiograms.

(3) There is, as a rule, no time relation between the characteristic points on the two types of curve, which shows that distinct physiological processes are responsible.

Translated by J. E. S. BRADLEY

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* There exists a translation into English of this work.

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FLUORESCENCE SPECTRUM MEASUREMENTS ON MODEL SYSTEMS AND LIVING OBJECTS USING PHOTOMULTIPLIERS*

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PRECISION spectrophotometric methods have latterly become widely used in biological studies [1]. Absorption and fluorescence measurements can be applied without disturbance to living systems, such as cell suspensions, leaves, roots and animal tissues. Many technical difficulties have to be overcome, however, since the concentrations of the materials of interest are usually small, and their absorptions are masked by the heterogeneous scattering and absorbing structures in the cell. Many

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biological processes are rapid, so special high-speed methods are required. Many of these difficulties can be overcome by using photomultipliers; Lundegardh [2] was a pioneer in this field, and studied the cytochrome systems of roots and leaves. Chance [3] developed an apparatus for recording differential absorption spectra which could be used to study the reaction kinetics of cytochrome systems and of mitochondria [4, 5]. Duysence and others [6, 7] have studied the rapid changes in the absorption spectra of photosynthesizing organisms on illumination. Precision measurements of the fluorescence spectra and quantum yields of pigments from leaves, algae, bacteria, etc. have been made using photomultipliers [8]. The fluorescence emission and excitation spectra of chlorophyll and of related pigments have been studied [9]. Photomultipliers used as quantum counters have been used [10, 11] to detect the afterglow of chloroplasts and the bioluminescence of seedlings [12].

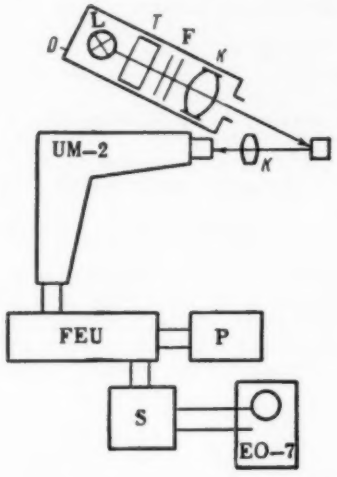

FIG. 1. Apparatus for measuring fluorescence spectra. *O*, illuminating system; *L*, quartz–mercury arc, type SVDSh-250; *T*, heat filter, *F*, light filter, *K*, condensers; UM-2, glass monochromator; FEU, photomultiplier; *P*, power supply; *S*, selective amplifier; EO-7, cathode-ray oscilloscope.

Fluorescence methods can also be used to determine the states of the pigments in the cell; in addition they give evidence on energy migration and the utilization of absorbed energy in reaction sequences.

We have used the apparatus outlined in Fig. 1 in our fluorescence studies. The air-cooled SVDSh-250 mercury arc is imaged on the object set up in front of the entrance slit of a UM-2 monochromator; the photomultiplier is placed behind the exit slit. The photomultiplier output is amplified and fed to an EO-7 oscilloscope. A medical-type PRK-2 lamp can replace the SVDSh-250; the light from the arc is appropriately filtered; the heat filter is a layer of water 5–7 cm thick. The objects (solutions, materials in evacuated Thunberg tubes, or leaves) were contained in holders allowing of cooling to −150°C. The selected FEU-17 multiplier had an overall sensitivity of 550 A/lumen at 750 V; the multiplier and amplifier were fed from BAS batteries. The fluorescent emission was, of course, modulated with the 100 c/s frequency

of the arc, so a double T-type filter, tuned to this frequency, was inserted in the amplifier as in Sushchinskii's system [13]; this system is widely used with photomultipliers [14]. In this case it reduced the noise level by a factor of 50.

The spectral sensitivity of the equipment was measured using a standard strip lamp fitted with a sector-disc chopper. The sensitivity was maximal at 540 mμ, and slight at 400 and 700 mμ, though the fluorescence maximum of chlorophyll in leaves (686 mμ) could be measured. Some typical spectra so obtained are given below. We have used the apparatus to study the fluorescence of riboflavine and reduced diphosphopyridine nucleotide of yeast cell suspensions and of leaves, and the concentration quenching of fluorescence in the fluorscein–haemoglobin system.

The fluorescence spectra of living objects

The fluorescence spectra of living cells in the blue and green regions of the visible spectrum have not been much studied because they are weak; this is partly caused by the low concentrations of materials which fluoresce in these regions. We have used *Saccharomyces fragilis* and bean and maize leaves, covering the 400–600 mμ range. Figure 2 shows the curve for the yeast, with maxima at 410, 420, 435 and 455 mμ.

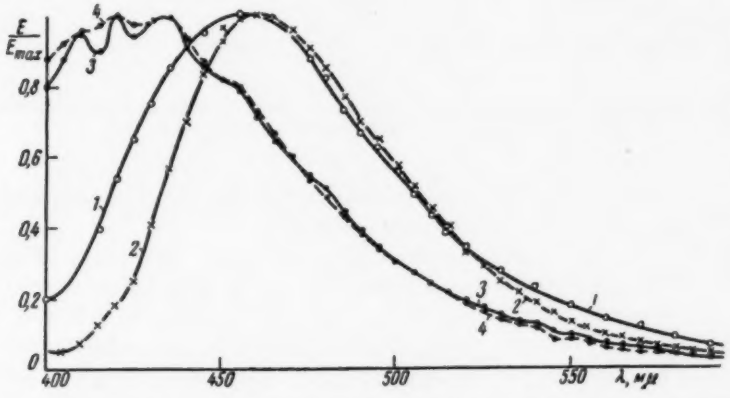

FIG. 2. Fluorescence spectrum, corrected for spectral variation of sensitivity in apparatus. (1) Reduced diphosphopyridine nucleotide (pH 7·0); (2) riboflavin in distilled water; (3) yeast (taken with 0·3 mm slit); (4) yeast (taken with 0·5 mm slit).

Figure 3 shows the spectrum from etiolated bean leaves cooled to −150°C; maxima are seen on a falling background at 410, 420, 435, 455 and 460 mμ. Green leaves show a similar spectrum, with maxima at 410, 425, 435 and 466 mμ, and a hump at 485–490 mμ. The general shapes of the spectra are the same at room temperature, but the intensities are much lower; there is a weak maximum at 550–555 mμ. These maxima (except that at 485 mμ) were also detected photographically (maxima at 635, 655 and 695 mμ were also found photographically).

The spectra of riboflavine and of reduced diphosphopyridine nucleotide were studied to identify the materials responsible for the leaf fluorescence*. The nucleotides

* The reduced diphosphopyridine nucleotide was prepared by reacting a DNP preparation (kindly presented by G. P. Brin) with thiosulphate.

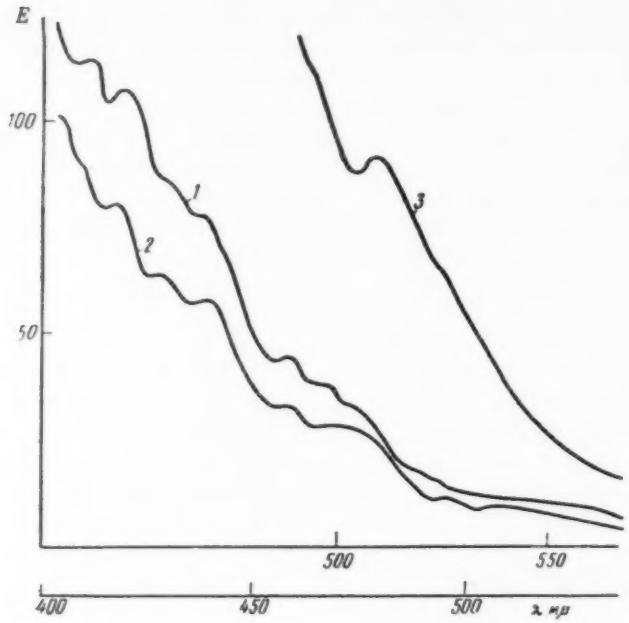

FIG. 3. Fluorescence spectra of etiolated leaves (corrected). (1) Rye, (2) bean, (3) section of curve (2) on larger scale (wavelengths read on upper scale).

gave a maximum at 460 mμ, and the riboflavine at 465 mμ. The solutions used were of low optical density, to eliminate reabsorption effects.

The results for yeast and reduced codehydrase agree with the published data [15-17]; the 435 mμ maximum found with yeast can be assigned to reduced diphosphopyridine nucleotide bound to protein [18]. The maxima at 435 and 460 mμ found with leaves are evidently due to DNP-H_2 in the bound and free states, while the weak maximum at 485–490 mμ in green maize leaves may be due to riboflavine. It is difficult to interpret the fluorescence spectra of the leaves in the blue–green region completely as yet, though the general shape of the curve indicates that it is the sum of many components. Fluorescence studies on the cellular components enable us to follow the processes in cells without disturbing them.

Concentration quenching in the fluorscein–haemoglobin system

Energy migration studies on model protein systems are of interest in relation to photosynthesis mechanisms. Sensitized photoreaction studies [19] and concentration quenching phenomena in protein–dye systems give us data on these mechanisms. Our apparatus could be used over wide concentration ranges with the fluorscein–haemoglobin system. Figure 4 shows the concentration quenching in equimolar (haem basis) mixtures of human haemoglobin and fluorscein in 0·1 N borate buffer at pH 9·2; the curve for fluorscein alone is given for comparison. Reabsorption effects were eliminated by irradiating from the front. The fluorescence spectrum is unchanged

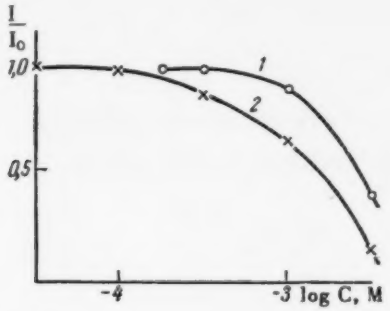

FIG. 4. Concentration quenching of fluorescence in fluorscein solutions (1), and in fluorscein solutions at pH 9·2 containing haemoglobin to give an equimolar content of haem, (2).

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from 4×10^{-7} to 5×10^{-5} M, so the concentration quenching is caused by energy migration from fluorscein to haem, and not by reabsorption by the energy acceptor. As the dye combines with the protein at concentrations in the 5×10^{-3} M range we can assume that here the energy migrates within and between protein molecules.

Translated by J. E. S. BRADLEY

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* There exists a translation into English of this work.

A MODIFICATION OF THE MICROELECTROPHORESIS CELL OF ABRAMSON AND MOYER*

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(*Received* 18 *November* 1957)

THE microelectrophoresis method is used widely in biological and chemical research. A large number of different chambers have been suggested for measurement of the velocity of migration of particles in an electrical field. Usually they take the form of closed cells of flat or cylindrical shape, which are attached to the stage of a microscope†.

As is well known, the observed electrophoretic velocity of migration of particles in such cells depends not only on the zeta-potential of the particles, but also on the electro-osmotic flow of fluid. In a closed system electro-osmotic flow of fluid creates a pressure which impedes the movement of fluid layers further away from the walls, and its middle layers are forced to move in the reverse direction. Consequently the velocity of liquid flow at different depths changes, not only in magnitude, but also in direction. The velocity of electro-osmosis, superimposed on the velocity of electrophoresis, gives an observed mobility of particles differing from the true mobility (which depends on the zeta-potential of the particle). The observed and true velocities of electrophoresis coincide in those cases where the fluid is stationary. In a flat, closed cell this condition is fulfilled at a depth equal to 0·211 of the height of the cell [1]. It follows that the velocity of electrophoresis of particles should be measured at such a level. However considerable difficulties arise in this case. At the indicated depth the velocity gradient dv/dy (where v is the velocity of migration of particles and y is the depth of the cell) is large. Therefore, even with slight deviations from the required observation depth, considerable errors are introduced in determining the value of v. In addition, as a consequence of the large value of the velocity gradient, at a depth of one-fifth of the height of the cell particles experience rotation during displacement which can lead to further errors.

Determination of the depth of observation in each experiment is easier to achieve in flat cells than in cylindrical ones. Therefore, in spite of a number of improvements of cylindrical cells, flat cells are preferable. Flat cells of shallow depth are used for observing fine particles, but the errors mentioned above are greatly increased. This requires that work be carried out with flat cells not less than 0·4–0·5 mm in height. Apart from this, high magnification is difficult and objectives must have an adequate focal length.

The shortcomings mentioned above induced us to make alterations to the flat cell. The flat cell of Abramson and Moyer [2] was selected as the starting point.

* Biofizika **3**: No. 5, 610–613, 1958.

† Chambers serving for measurement of the displacement of a falling drop have not found wide acceptance and are not considered here.

THE PRINCIPLE AND THEORY OF OPERATION OF THE MODIFIED CELL

To remove the defects enumerated above, it is necessary to arrange conditions in the cell such that the fluid is static at its centre. This is achieved by adding a tube of fixed dimensions parallel to the cell. The tube attached in parallel, which contains the fluid under study, being in the same electrical field, regulates the fluid pressure at the ends of the cell so that the flow of the middle layers of fluid in the cell is arrested. The movement of the fluid of a cell having a tube in parallel to it is represented diagrammatically in Fig. 1.

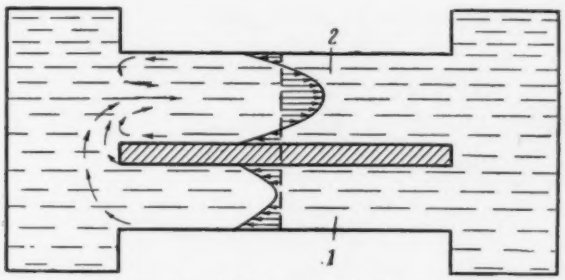

FIG. 1. Diagram of the propagation of fluid currents in the modified microelectrophoresis cell. (1) Cell; (2) parallel tube.

The problem reduces itself firstly to finding a difference of pressures at the ends of the cell which would render static the fluid in the centre of the cell; secondly, to deducing the relation between the pressure difference found and the displacement of fluid in the cell Q; and finally, to determining the dimensions of the parallel tube which at such a pressure difference would ensure a fluid displacement Q, equal to the fluid displacement in the cell.

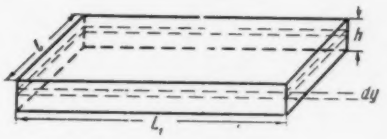

FIG. 2. Drawing of the cell.

Let us examine firstly the movement of fluid in the cell (Fig. 2). The following notation is employed : L_1 = length, h = height*, l = breadth, y = depth (variable) of the cell. In the centre of the cell $y = 0$, and at the walls $y = \pm h/2$, v = velocity of movement of the fluid, p = difference of pressures at the ends of the cell preventing movement of fluid in the centre of the cell, η = viscosity of the fluid.

For laminar flow of fluid in the cell

$$\frac{d^2v}{dy^2} = \frac{P}{\eta L_1} dy. \tag{1}$$

(Here the pressure P is the same in all sections of the cell.)

* Here we calculate the distance h not from the solid–liquid interface, but from the shearing plane of the electrical double layer.

Then :

$$v = \frac{P}{2\eta L_1} y^2 + Ay + B.$$

The limiting conditions are: $y = 0$, and $dv/dy = 0$ when $v = 0$; and $y = \pm h/2$ when $v = v_{max}$, where v_{max} is the velocity of electro-osmosis.

With $y = 0$ from the limiting conditions $A = B = 0$, then:

$$v = \frac{P}{2\eta L_1} y^2.$$

With $y = \pm h/2$, from the limiting conditions:

$$v_{max} = \frac{Ph^2}{8\eta L_1} \text{ and } P = \frac{8\eta L_1 v_{max}}{h^2}. \tag{2}$$

The fluid displacement for the cell is given by:

$$Q = \int_{-h/2}^{+h/2} vldy = \frac{Plh^3}{24\eta L_1},$$

or, substituting P from (2)

$$Q = \frac{lhv_{max}}{3}. \tag{3}$$

Let us examine now the movement of fluid in the parallel tube (Fig. 3). The following nomenclature is employed: L_2 = length, R = radius (internal)*, r = radius (variable) of the tube. The limiting conditions are: $r = 0$ when $dv/dy = 0$; and $r = R$ when $v = v_{max}$, where v_{max_1} is the velocity of electro-osmosis.

With laminar flow

$$d\left(r \frac{dv}{dr}\right) = \frac{P}{\eta L_2} rdr,$$

hence

$$v = \frac{P}{4\eta L_2} r^2 + ALnr + B,$$

from the limiting conditions $A = 0$ and

$$v_{max_1} = \frac{P}{4\eta L_2} R^2 + B,$$

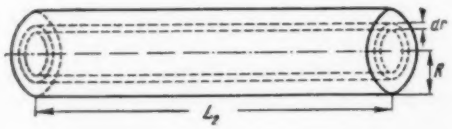

FIG. 3. Diagram of the tube.

from which

$$B = v_{max_1} - \frac{P}{4\eta L^2} R^2.$$

* Here for radius R we take the distance from the axis of the tube to the shearing plane in the electrical double-layer at the walls of the tube.

Thus,

$$v = v_{\max_1} - \frac{P}{4\eta L^2}(R^2 - r^2).$$

We can find the fluid displacement:

$$Q = \int_0^R v 2\pi r dr = \pi R^2 v_{\max_1} - \frac{\pi R^4 P}{8\eta L_2}.$$

Under the conditions of the problem it is necessary that P and Q for tube and cell should be equal.

We can substitute the value P for the cell from (2), then

$$Q = \pi R^2 v_{\max_1} - \frac{\pi R^4 L_1}{L_2 h^2} v_{\max}. \tag{4}$$

We can equate displacement for the cell from (3) with displacement for the tube (4):

$$\frac{lhv_{\max}}{3} = -\pi R^2 v_{\max_1} + \frac{\pi R^4 v_{\max}}{h^2}\frac{L_1}{L_2}.$$

If tube and cell are made of the same material, then

$$v_{\max} = v_{\max_1}$$

and then

$$\frac{L_1}{L_2} R^4 - h^2 R^2 - \frac{lh^3}{3\pi} = 0. \tag{5}$$

From equation (5) it is easy to find the required relationship of the dimensions of cell and parallel tube.

With $L_1 = L_2$

$$R = \frac{h}{2} \pm \left[2 + 2\left(1 + 0{\cdot}425\frac{l}{h}\right)^{\frac{1}{2}}\right]^{\frac{1}{2}}. \tag{6}$$

The condition of absence of flow of fluid in the centre of the cell will be fulfilled independently of the potential gradient of the external electrical field and the viscosity of the solution as shown by equation (5).

The cell and the tube must be made from the same material. It is convenient to mount them as shown in Fig. 4. Our cell, constructed according to the requirements of equation (6), had the following dimensions: thickness of upper and lower walls $d = 0{\cdot}03$ mm, height $h = 0{\cdot}1$ mm, breadth $l = 1{\cdot}3$ mm, internal diameter of tube $D = 0{\cdot}264$ mm, the length of the cell L_1 and the length of the tube L_2 were both 30 mm.

Control studies with rat erythrocytes demonstrated the suitability of the cell for measuring electrophoretic mobility of cells. Erythrocyte mobility, determined at the middle depth, coincides with published results [3]. It does not depend on the potential gradient of the external electrical field or on the viscosity of the fluid. The relation of observed velocity of electrophoresis of erythrocytes to cell depth is given in Fig. 5.

As can be seen from Fig. 5, slight errors in depth determination do not substantially affect measurement of the electrophoretic mobility of particles (as compared with Abramson and Moyer's cell). Determination of the depth at which measurement should be carried out (half way up the cell) can itself be checked by the fact that the

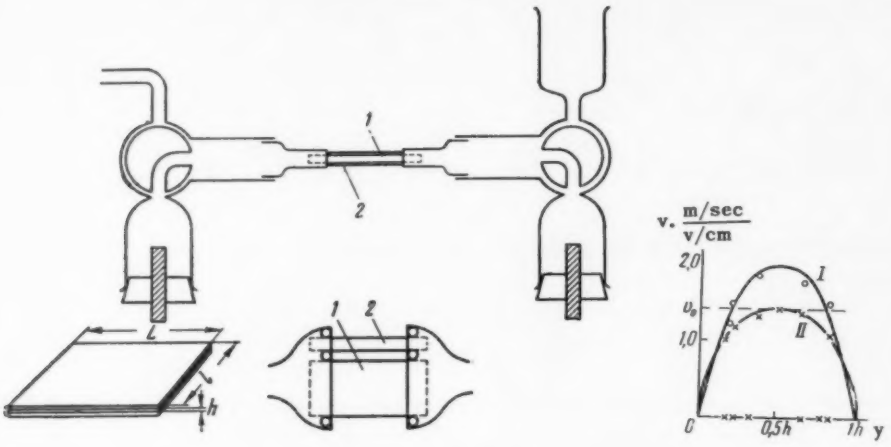

FIG. 4. (1) microelectrophoresis cell; (2) parallel tube.

FIG. 5. Ratio of the observed electrophoretic velocity of rat erythrocytes to cell depth. I—Abramson's cell; II—modified cell; v_0—true velocity of electrophoresis.

observed velocity of particle migration is at a maximum at this depth. In an ordinary cell such a check is impossible, since at the depth of measurement (0·21 of the height) the velocity is not at an extreme value. Because of the small velocity gradient at the level of observation, i.e. at the centre of the cell, rotation of particles during migration does not occur. All this makes the cell convenient to operate and allows a reduction in its total depth. Likewise the magnitude of errors due to possible convection currents is reduced and measurements can be carried out on small objects.

I convey my profound thanks to I. E. El'piner and M. M. Bongard for their attention and help in this work.

Translated by C. R. PRINGLE

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METHODS OF STUDYING THE CHANGES IN THE RESTING POTENTIALS OF SINGLE MUSCLE FIBRES*

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MICROELECTRODES have become widely used in the last 10 years for recording the resting and action potentials of single cells. Graham and Gerard [1], followed by Ling and Gerard [2–5] and Nastuk and Hodgkin [6], were the first to study single fibres from transversely striated frog muscle in this way. Many others subsequently used the method with many different tissues. The resting potential is usually measured as the mean from repeated brief tests of the potential difference between the cell and the external medium. The results are worked up statistically; the extreme values are rejected. Table 1 shows that the average values given for single frog sartorius muscle fibres by different workers disagree.

TABLE 1. RESTING POTENTIALS OF NORMAL FROG SARTORIUS MUSCLE

Year	Authors	No. of fibres	Mean resting potential (mV)	σ	Extreme values (mV)
1946	Graham and Gerard [1]	65	61·8	—	41·0–80·4
1949	Ling and Gerard [2]	1350	78·4	±5·3	—
		206	97·6	±5·7	—
1950	Nastuk and Hodgkin [6]	161(18°)	88·0	±1·0	82·0–99·0
		31(7°)	84·0	±2·0	79·0–90·0
1951	Fatt and Katz [7]	—	90·0	—	—
1953	Jenerick and Howard [8]	—	82·0	±0·6	—
1953	Nastuk [9]	60	92·8	±0·5	—
1956	Adrian [10]	296	92·2	±0·5	87·9–95·3
1957	Kostiuk [11]	300	83·0	—	73·0–117·0

Our aim was to study the methods in detail and to find how one might use a single fibre with an electrode in it for a long period. We used sartorius muscle from *Rana temporaria* during the autumn, winter and spring. The muscles were placed with their internal surfaces upwards in a chamber containing flowing Ringer made up from Ling and Gerard's recipe [2], but with rather less potassium (NaCl 6·5, $CaCl_2$ 0·14, $NaHCO_3$ 0·20, and KCl 0·12, all in g/l.). The Ringer flowed evenly at about 1 drop/sec and formed a layer 1–2 mm deep on the muscle. The design of the microelectrodes has been described previously [12]. The electrodes were

* Biofizika **3**: No. 5, 614–619, 1958.

filled with 3 M or 118 mM KCl; the outer surfaces were carefully flushed free of KCl and dried on filter paper before use.

A single-valve electrometer of input resistance $1{\cdot}28 \times 10^{10}$ was used (Dubridge–Bardt circuit—Fig. 1) with a 1E1P electrometer tetrode of grid current less than 7×10^{-14} A. The input lead was ceramic insulated. The output to the galvanometer was fitted with a shunt-type filter to eliminate the alternating component. All resistors except the grid leak were wire-wound; the circuit was carefully screened. The drift rate on stabilized supplies after 3 hr running was less than 0·2 mV/hr. A type M 25/6 galvanometer was used; the overall response was 13 μV/mm at 1 m. The sensitivity used was usually 1/10 to 1/30 of the maximum.

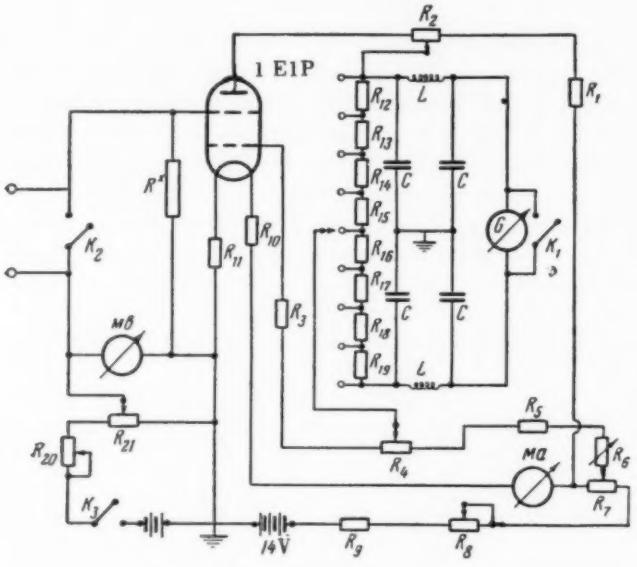

FIG. 1. The valve electrometer circuit.

The zero was checked before each reading and could be adjusted using the resistors R_2 and R_4, with the switch K_2 closed. The balancing circuit included a type M 80/1 multirange meter. Two methods of reading could be used, namely from the galvanometer (without balancing) and from the millivoltmeter in the balancing circuit. Quite small changes (about 10^{-5} V) could thereby be detected in fairly large potential differences (about 0·1 V).

The microelectrodes were at first connected via screened glass tubes with their ends filled with agar made up in 3 M KCl and with platinum spirals to make contact with the agar (Fig. 2*A*). These were subsequently replaced by calomel half-cells (Fig. 2*B*). The microelectrodes were inserted vertically into the muscle with MM-1 micromanipulators. The comparison electrode had a broken tip and was filled with KCl solution; it was inserted in the Ringer at the end of the chamber.

In all, 46 fibres from 17 muscles (Nos. 1–17) gave resting potentials with a mean of $70{\cdot}6 \pm 8{\cdot}7$ mV during the period 25 October to 26 December 1956. Electrodes of

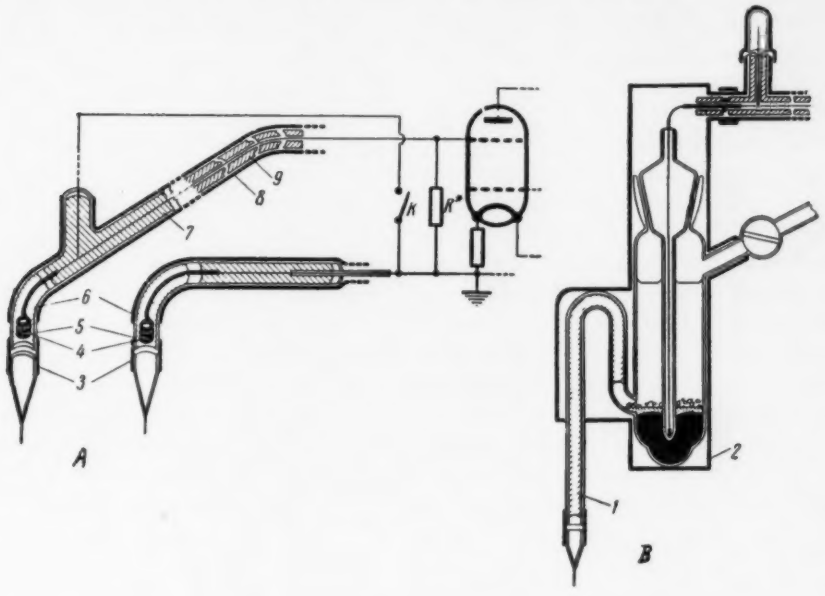

FIG. 2. Electrode system. (*A*): system in which polarization effects can occur, but which is suitable for some purposes (*B*): calomel half-cell system, polarization impossible. (1) and (6) KCl agar; (2) and (8) screening; (3) rubber sleeves; (4) platinum spirals; (5) glass; (7) ceresin wax; (9) porcelain.

diameter less than 3 mμ made of molybdenum glass were used in this series (input as of type *A* Fig. 2). In the second and all subsequent series the type *B* input was used; polarization effects were thereby eliminated. The second series was run from 5 February to 20 April 1957 and covered 52 fibres from 11 muscles (Nos. 18–28); the mean resting potential was 75·7 $\pm$ 8·6 mV.

Pyrex glass was used to make the electrodes for muscles after No. 20, because thin electrodes can more readily be made. The molybdenum glass also gave electrodes of rather low resistance. Repeated washing sometimes improved the resistance, possibly by removing a conducting surface layer. The molybdenum glass electrodes may have distorted the resting potentials, particularly if the membrane is responsible for the potential, as on Goldman's theory [13, 14]. Nevertheless it was on occasion possible to keep a molybdenum glass electrode in a single fibre for over 2 hr without affecting the potential appreciably. The pyrex electrodes could be used for longer times; with No. 22 (using an electrode 2·4 mμ in diameter filled with 3 M KCl) the resting potential fell from 90 mV to 76 mV in the first 200 min (in the next 16 min it fell to 16 mV). An adjacent fibre in the same muscle showed a fall from 93 mV to 90 mV in 65 min, after which the test was ended. Two other fibres in this muscle gave potentials of 86 and 89 mV; the mean for the four was 89·5 $\pm$ 1·9 mV.

The time and potential depend very much on where the electrode is inserted in the fibre; in some cases the potential reaches a steady value as soon as the electrode penetrates within the muscle, whereas in others it rises slowly. In the first case a

fibre is entered radially, since the electrode can be moved through distances of 40–80 mμ, i.e. right across the fibre, without affecting the potential appreciably. The initial potential then persists for a long while. In the second case the slightest movement alters the potential; here the electrode merely damages the fibre, or enters it tangentially. If the potentials were in fact injury potentials caused by the proteins liberating bound ions [15–16] the potentials should vary in both cases. Not only so, but the ions would soon be removed, having no specific interfaces to retain them, and so the potentials would fall very much more rapidly than is actually found.

Our slow recording system (galvanometer time-constant 6·5 sec) could not, of course, record how the potential rose as the electrode penetrated, but users of cathode-ray oscilloscopes have found that the rise is abrupt. This indicates that the potential difference actually arises at the surface of the cell, and not from damage.

The rest of the series were done on warm days with frogs which had been kept in a terrarium at room temperature. The animals were very flabby, with whitish opalescent or dark-red muscles. The mean resting potentials found were much lower than those found with the autumn and winter frogs. From this group, 30 fibres from 7 muscles (Nos. 29–35) gave a mean of 52·5 $\pm$ 10·7 mV (25 April to 14 May 1957). Other frogs kept in the terrarium, but under better conditions, gave a mean potential of 67·9 $\pm$ 5·1 mV from 14 fibres from 5 muscles (Nos. 36–40, 17 May to 3 June 1957). The muscles from the flabby frogs gave very marked differences between fibres from the surface and from the interior of the muscle; others have found the same difference. In No. 35 the surface fibres gave 40, 48, 51 and 44 mV, while the deeper ones gave 66·7 and 67·5 mV. This explains why the root mean square deviation for the muscle as a whole was so large in this set: values for No. 35 (seven fibres) and No. 38 (four fibres) are $\pm$ 11·5 and $\pm$ 14·0 mV respectively.

The Ringer cannot replace the lymph completely, since it supplies no materials to provide the energy requirements of the muscle [5], which must therefore draw on its reserves. The surface fibres are in contact with the Ringer; their potentials fall more rapidly, particularly if the muscle is taken from a flaccid frog.

Adrian [10] has shown that almost all electrodes filled with 3 M KCl show an inherent electrode potential, which can be detected by inserting the electrode in Ringer. The electrode potential increases with the resistance and of course vanishes if the tip is broken off. There is no simple relation between the potential and the resistance (mainly controlled by the tip diameter). The potential can be comparable with the resting potential, so the measured potential is the resultant of the true resting potential and of the electrode potential, neither of which can at present be determined separately with any accuracy [6, 10, 11]. The electrode potential depends on the concentration and (probably) ionic composition of the solution in which it is inserted. Adrian [10] has shown that the potentials of all electrodes filled with 3 M KCl increase as the concentration of the solution falls and are always negative under these conditions. A solution 3 M in KCl gives the minimum electrode potential. Adrian and Kostiuk [11] have tried to minimize the component due to the electrode potential, Adrian by using only electrodes with potentials of from 0 to -5 mV (against Ringer); this involved rejecting some 75 per cent of otherwise suitable electrodes.

The electrode potential depends on the concentration gradient between the solutions inside and outside the electrode; our apparatus was such as to enable us to

use electrodes of very high resistance, so we reduced the concentration of the filling, and hence the electrode potential. Figure 3 shows how the electrode potential depended on the KCl and NaCl contents of the outside solution when the filling is 118 mM KCl (a concentration corresponding osmotically to Ringer). The potential is almost zero when the outside solution is somewhat more concentrated than the filling; this ought rightly to occur when the concentrations are equal. The concentrations are in fact equal, because adsorption in the very fine capillary reduces the concentration. The effects may be partly related to the ionic composition of the glass [17] relative to the external solution of KCl (or NaCl), even though pyrex contains relatively little potassium (1·00–1·74 per cent by weight) and sodium (3·68–4·00 per cent by weight). The walls of the capillaries act like glass membranes, since they are seldom more than 0·1 mμ thick. This may explain why the resistance and electrode potential tend to show the same trend, because the lower the resistance of the capillary the more the membrane is shunted, and hence the lower the electrode potential. Electrokinetic effects must also play a part, particularly since the electrolytes of differing concentrations come into contact via a very fine capillary.

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It might seem that the electrode potential could be entirely eliminated by filling the electrode with a solution of concentration the same as is found in the myoplasma; the total ion concentration is required for this purpose. Approximate values are 139 mequiv K and 15 mequiv Na/kg [10, 14] of free water (total 154 mequiv), so it might be thought that the required concentration is known roughly (see the curves in Fig. 3). We cannot assume that all the ions in a cell are free, though; nor are the activity coefficients known. The content of chloride ions (1·2–2·4 mequiv/kg of free water [14]

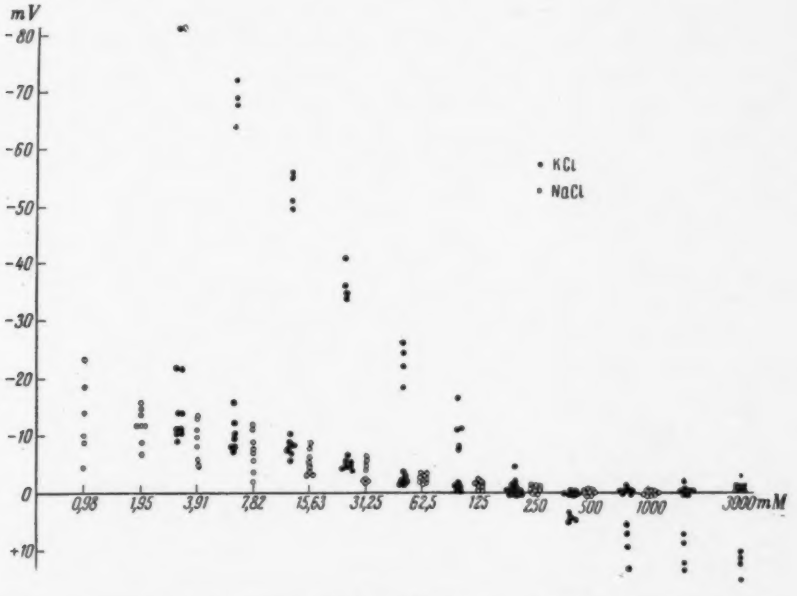

FIG. 3. Contact e.m.f.s for four microelectrodes filled with 0·118 M KCl vs. concentration of solution. ●–KCl, ○–NaCl.

is also not such as to balance the cations; the osmotic pressure due to the colloids has not yet been accurately measured, either. Osmotic equilibria are affected by electrochemical potential gradients (Donnan equilibria), so there are difficulties of a theoretical nature in measuring total free ion concentrations in cells.

If we assume that the intra- and extra-cellular fluids are in osmotic equilibrium, the total ion concentrations on the two sides of the cell membrane must be the same, so the difference in electrode potentials produced by the two fluids should be small. If the partial ion concentrations (particularly of Na^+ and K^+) are equal as well, the electrode potential should be unchanged by shifting the contact from one fluid to the other. This could occur if no bound ions were freed when the electrode penetrates the cell; we could then subtract the constant electrode potential from the measured potential difference and get the true resting potential.

Whether such subtraction is justified or not, we have subtracted the electrode potentials from the measured potential differences in all cases; this may explain why we have found mean resting potentials lower than those given by others.

Woodbury *et al.* [18] have shown that the measured resting potential and the external diameter of the microelectrode are inversely related; the reason may be that larger electrodes do more damage. The electrode potential, which increases as the diameter of the electrode decreases, may play a large part here. It may be that the relatively large electrodes we used in the first series are responsible for the relatively low resting potentials found. Our experience indicates that the scatter in the mean resting potentials given by various workers for frog sartorius muscle arises from many points of technique in making the electrodes and from physiological variations in the animals.

The resting potential remains the same for long periods when appropriate conditions prevail. With muscle No. 14, examined on 14 September 1957 (with a 1·5 mμ electrode filled with 118 mM KCl, of resistance 163·4 Ω), the potential fell from 84 mV at the start to 81 mV after 7 hr 25 min (the end of the experiment).

The mean values should not be used in studying changes in resting potentials; values obtained directly on the fibres being studied are much to be preferred, as giving less ambiguous results.

Translated by J. E. S. BRADLEY

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THE USE OF ULTRA-HIGH FREQUENCIES IN BIOLOGICAL RESEARCH*

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The electrical methods of studying the circulation of blood, available at the present time, are based on the recording of alterations of the electrical parameters of the tissues of an organism. Electroplethysmography [1], rheography [2], impedance plethysmography [3, 4] and dielectrography [5] are some of these methods. In using these for recording change of electrical parameters, electrodes are placed on the tissue which is being studied and an alternating voltage of low or medium radio frequency (up to 200–300, and sometimes even 1000 kc/s) is applied to them.

The necessity of placing the electrodes on the region under study limits the use of the methods indicated above to observations on animals under acute and chronic experimental conditions. Attempts to use these methods for observation of human blood circulation [3, 4, 6], by placing electrodes on the skin, have not been very effective, in view of the high resistance of the skin and the impossibility of knowing the magnitude of the changes in electrical conductance between the electrodes due to cutaneous blood-vessels. They have indicated merely that it is possible to use these methods in recording the reactions of blood-vessels in the extremities [6, 7] and to study the rate of propagation of the pulse wave [8].

It is possible to extend the electrical methods of investigating blood circulation by making use of currents of ultra-high frequency (u.h.f.) (above 500 Mc/s) whose physical properties enable a "contactless" method of studying blood circulation to be devised.

A way of devising a contactless method follows from an examination of the u.h.f. electrical parameters of living tissues. The human and animal body is semi-transparent to radio waves in the u.h.f. waveband, since the imaginary permitivity component of living tissues is considerably smaller than its real portion. A plane electromagnetic wave, falling normally on any part of the body, will be partially absorbed in it. The magnitude of absorption depends on the average values of the dielectric constant (ϵ) and the conductivity (σ) through the tissues of the organism lying in the path of the radio waves. If the quantitative proportions

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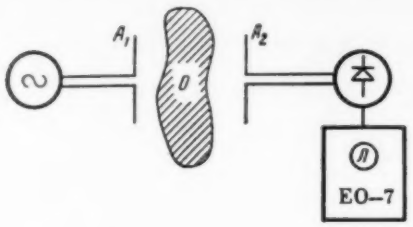

FIG. 1. The principle of recording electrical parameters of living tissues at ultra high frequencies. *O* is the object.

between any tissues of different dielectric constants and conductivities change in the part of a body through which radio waves are passing, then ϵ_{av} and σ_{av} increase or decrease correspondingly, and consequently the absorption of the radio waves changes. All media of an organism, differing in values of ϵ and σ, can be represented conveniently as two groups, alteration of the quantitative proportions of which determine the different absorption of radio waves in a given region. Mobile media (blood, spinal fluid, air in the lungs), whose amount is not constant in a given region, can be segregated into the first group, and all remaining media (muscles, fat and bone tissues) into the second group.

An apparatus for recording change of radio-wave absorption (Fig. 1) can be represented as a transmitting antenna A_1, radiating predominantly in the direction of the object under study, and receiving antenna A_2 picking up the signal passing through the object of study O.

From electrical circuit theory this system can be represented in the form of an equivalent quadripole (Fig. 2*a*), where Z_A and Z_B are the input impedances of the receiving and transmitting lines, and where the impedance Z_{AB} depends on the properties of the external medium with constant distance between the antennae.

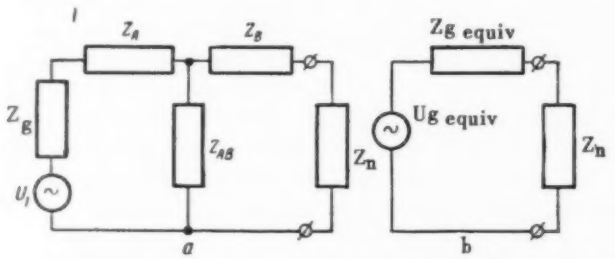

FIG. 2. The equivalent circuit in the contactless method of studying blood circulation.

According to Thévenin's theorem such a quadripole is equivalent to another system (Fig. 2*b*) in which the load is connected directly to a generator with an electromotive force (e.m.f.) equal to

$$U_{\text{equiv}} = \frac{U_1 Z_{AB}}{Z_g + Z_A + Z_{AB}}, \tag{1}$$

and with an internal impedance

$$Z_{\text{equiv}} = Z_B + Z_{AB}. \tag{2}$$

From equations (1) and (2) it is seen that the e.m.f. and the internal impedance of the equivalent generator are determined by the properties of the external medium, i.e. by its electrical parameters, moreover the e.m.f. of the equivalent generator is inversely proportional to the absorption of electromagnetic energy in the object under study, and the presence of the object close to the receiving system, expressed in an alteration of directivity and internal resistance of the receiving antenna, affects the internal impedance of this generator. These relations determine the size of the signal in the receiving system when a change occurs in the quantitative proportions of media of groups I and II in the part of the body which is being studied.

To find the maximum conditions of sensitivity of the contactless method it is necessary to examine the role of each of the factors, apparent from an examination of the equivalent circuit, which affect the level of signals in the receiving system.

The absorption of radio waves in the object which is being studied is characterized by the ratio of the power incident on the object to the power which passes through it, and is defined by the formula:

$$P_{\text{in}} = P_{\text{thru}} \times e^{2a_{\circ} z}, \tag{3}$$

where P_{in} is the power incident on the object; P_{thru} is the power passing through it; $a_{\circ}$ is an attenuation constant equal to

$$(\epsilon_{\text{av}}^2 \mu^2 + \sigma_{\text{av}}^2 \mu^2 \omega^2 \sin \varphi/2)^{\frac{1}{2}}, \tag{4}$$

ϵ_{av} and σ_{av} are the average values of the dielectric constant and conductivity of the experimental object; μ is the thickness of the object; ω is the frequency of the applied electromagnetic field; φ is a tabular function:

$$\varphi = \tan^{-} \frac{^{1}\sigma_{\text{av}}}{\epsilon_{\text{av}}\ \omega}\ . \tag{4a}$$

According to formulae (3) and (4), in a constant frequency electromagnetic field, radio-wave absorption is determined by the average values of the dielectric constant and the conductivity of the tissues present in the space between the antennae.

Since in the u.h.f. waveband the dielectric constant and the conductivity of live tissues change in relation to the frequency of the electromagnetic field applied, the maximum alteration of their average values will also be observed in that portion of the waveband at which the difference between the electrical parameters of tissues of groups I and II will be greatest.

According to the theory of Debye [9], alteration of the electrical parameters of living tissues is caused by resonance absorption of the electromagnetic field by polar molecules of the tissues (mainly by water and protein molecules), when the frequency of the inherent oscillations of these molecules is somewhat less than that of the applied field. The period of the oscillations of an electromagnetic field, at which maximum absorption of power takes place, is expressed, according to Debye, by the formulae

$$\omega_m = \frac{12\ k\ T}{\eta a^3}\ , \tag{5}$$

where k is Boltzmann's constant; T is the absolute temperature of the medium; a is the radius of the molecules; η is the viscosity of water.

For water molecules at room temperature $\omega_m = 2{\cdot}8 \times 10^{12}$ sec^{-1}, and for protein molecules $\omega_m = 6 \times 10^{7}$ sec^{-1}.

Debye's theory predicts that the electrical parameters of any media change in a certain sector of the radio-waveband spread out to the right and left of the maximum absorption frequency. For homogeneous media the ratio of the boundary frequencies at which their electrical parameters alter is equal to 100.

As a consequence of the non-uniform structure of living tissues change of their electrical parameters is observed in the centimetre waveband (resonance of water molecules) and in the decimetre–metre waveband (resonance of protein molecules).

Besides the resonance absorption of radio waves, predicted by Debye's theory, a number of zones of maximum absorption of radio waves exist in which the quantum energy of the electromagnetic field corresponds to one of the possible energy levels of a protein molecule. However, these zones have no practical value in the application of the contactless method of studying blood circulation.

The magnitude of the change in the electrical parameters of blood in the zone of resonance absorption of radio waves can be calculated from Danzer's equations [10]:

$$\epsilon' = \frac{\epsilon_s - \epsilon^*}{1 + \omega^2 T^2} + \epsilon^* . \tag{6}$$

$$\sigma' = \sigma_s + \frac{\epsilon_s - \epsilon^*}{1 + \omega^2 T^2} \quad \frac{\omega^2 T}{4\pi} , \tag{7}$$

where ϵ' and σ' are the electrical parameters of blood in the dispersion region; ϵ_s and σ_s are their values in a direct current; and ϵ^* is the dielectric constant of blood at very high frequencies. Verification of these equations [11–13] has confirmed their sufficient agreement with experiment.

For group II tissues it is difficult to calculate the value of the electrical parameters in the dispersion region in view of the complexity of their structure. Therefore, for these, results of measurements of ϵ and σ in the u.h.f. waveband will be limited (see Table 1).

TABLE 1. VALUES OF ELECTRICAL PARAMETERS OF TISSUES

Tissue	Wave-length (cm)	t (°C)	ϵ'	ϵ''	ϵ	σ	σ_s	Author
Blood	230	36	—	—	—	—	0·00325	Bateman [14]
	17	35	56·2	18·1	—	—	0·014	Cook [13]
	10	35	56·0	15·9	—	—	—	Cook [13]
	3·18	35	47·8	19·7	—	—	—	Cook [13]
	1·27	35	30·2	26·0	—	—	—	Cook [13]
Spinal fluid	321	—	—	—	82	0·0136	0·023	El'piner [15]
	43	—	79·5	—	86	—	0·022	Beliakova
	10	—	70·0	22·5	—	—	—	Presman [16]
	3·18	—	57·5	24·0	—	—	—	Presman [16]
	1·27	—	16·5	29·0	—	—	—	Presman [16]
Muscle	230	—	—	—	—	—	0·0064	Bateman [14]
	10	—	51·0	18·0	—	—	—	Presman [14]
Brain	230	—	—	—	—	—	0·0038	Bateman [14]

The dispersion curves of the electrical parameters of tissues of groups I and II are shown in Fig. 3. From this material it is seen that the maximum difference of electrical parameters between tissues of groups I and II, lying in the 15–85 cm wave-length range, is not critical as regards frequency.

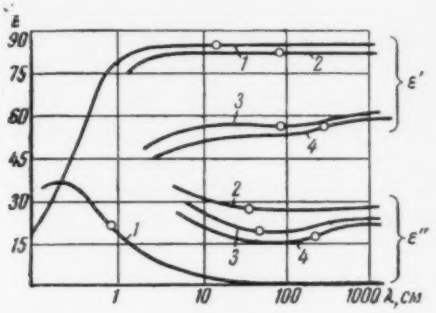

Fig. 3. The dependence of the change in the real and imaginary components of the dielectric constant of the media of an organism with wave-length. (1) water; (2) spinal fluid; (3) blood; (4) muscle.

In selecting a working wave-length, it is necessary to take into account the commensurability of the distances of the antennal systems from the object under study. For the method to be sufficiently effective the antennae must completely cover the object which is being studied. This circumstance limits the largest working wave-length to 80 cm. The lower limit of the working wave-length depends on the magnitude of the absorption of electromagnetic energy in the material under study. On the basis of formula (4), with constant ϵ and σ, radio-wave absorption increases with decrease of wave-length. This in its turn requires an increase in the power radiated by the transmitting antenna in order to provide a sufficient electromagnetic field intensity in the receiver. The relative biological effect of radio waves, of which the dependence on frequency coincides with the curve of tan δ, and reflection of radio waves from the surface of the body, also increase with increase of radio-wave absorption. From these considerations 30 cm must be considered as the lower limit of the working wave-length,

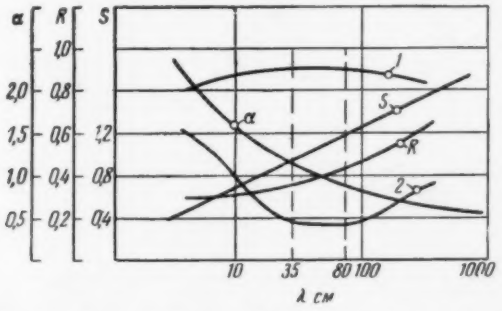

FIG. 4. Chart explaining selection of the wave-length range used in the contactless method. (*a*) coefficient of absorption; (*R*) coefficient of reflection; (*S*) magnitude of the ratio of the dimensions of the object under study to the transmitting antenna. (1) the difference of the electrical parameters of media of groups I and II; (2) the relative biological effect of radio waves.

where the absorption constant of radio waves, a_o, does not exceed unity, and the relative biological effect is comparatively small.

The second condition which, according to the equivalent circuit, determines the sensitivity of the contactless method, is the effect of the object under study on the parameters of the receiving antenna. To explain this relation, it is convenient to make use of an electrodynamical concept, according to which a half-wave electrical oscillator absorbs a certain quantity of energy from an incident radio-wave beam equal to that which falls on a surface of area $3\pi\lambda^2/8$. Since the numerical value of this surface is of the same order as the surface of the object under investigation, the effect of the latter on the receiving antenna can be considered as the problem of the effect of a passive dipole—the equivalent experimental object—on the characteristics of an active oscillator, i.e. the receiving antenna. In general, the length of this dipole is not equal to $\lambda/2$.

The equivalence of the experimental object to a passive dipole in its effect on a receiving antenna will show a good fit only where the distances between them are great. However, this analogy gives a qualitative representation of the effect of an experimental object on the characteristics of a receiving antenna. Graphic solution of such a problem is shown in Fig. 5. Changing the electrical parameters of the object under study in a given case is equivalent to changing the length of a passive dipole, which affects the directivity diagram (Fig. 5, *1*, *2*, *3*), and the radiation resistance of the active oscillator (Fig. 5, *4*, *5*, *6*). From these relationships it follows that the sensitivity of the method increases in the case where its action, together with alteration of radio-wave absorption, is monophasic, i.e. if an increase of radio, wave absorption causes simultaneously a loss of directivity and of matching of receiving antenna and feeder cable, while a reduction in absorption causes the reverse. The size of this effect (the difference in gradient of curves *1*, *2*, *3*, and *4*, *5*, *6* in Fig. 5) depends on the distance between the object and the receiving antenna.

Consequently a condition of maximum sensitivity of the contactless method, with corresponding matching of receiving antenna and feeder cable, can be achieved by selecting an optimum "object–receiving-antenna distance". (The radiation resistance

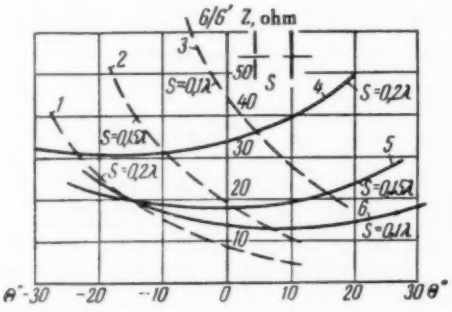

FIG. 5. The effect of a passive dipole on the characteristics of a receiving antenna. (G/G') the directivity coefficient of the antenna; (Z) the radiation resistance of the antenna; (S) the distance between antenna and passive dipole; ($\theta°$) the length of the passive dipole, expressed as electrical degrees of the displacement angle of current and voltage on right and left of a resonance line equal to $\lambda/2$.

of the antenna must be 8–10 per cent above the wave impedance of the feeder cable.)

From Fig. 5 it follows also that disturbance of the position of the object under study relative to the receiving antenna causes an alteration in the parameters of the latter, independently of the parameters of the object. Consequently this distance must be kept constant during observation.

On the basis of the data considered above, an apparatus was devised and constructed, consisting of a 2 W generator in the form of an earthed anode push—pull circuit, a transmitting antenna and a receiving system of a dipole-fed co-axial screened lead. The voltage from the lead is detected and fed to an EO-7 electron oscillograph. In order to avoid the necessity of using special direct-current amplifiers, the electromagnetic oscillations given out by the transmitter were modulated using a voltage of 50 c/s frequency. This allowed the low-frequency range of the EO-7 oscillograph to be used as an amplifier. The recording of alteration of radio-wave absorption in the space between the antennae was carried out photographically, by means of a device transporting a strip of film across the field at uniform rates of 10 and 30 mm/sec.

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By experiment it was determined that the maximum sensitivity of the method is achieved with a transmitting-antenna–object-distance of 10–15 cm, and an object–receiving-attenna distance of 0·5–1·0 cm. In this case the main portion of the electromagnetic energy passes through the body in the form of a circle with a diameter of 5–7 cm, making it possible to record changes in the blood content of such organs as the human heart, lungs, spinal cord, etc.

When recording changes in the blood capacity of deep-seated tissues the projection of the organ under study on the surface of the body must be parallel to the plane of the transmitting antenna, since, in the event of radio waves falling at an inclined angle, a portion of the electromagnetic energy is reflected, and at angles above 60° complete reflection occurs. The latter circumstance allows the area of study to be determined more exactly by definite orientation of the object under investigation in respect of the antennae.

Successful application of the contactless method for studying deep-seated organs depends on the extent to which it is possible to estimate the effect of changes in the blood content of "extraneous" tissues lying in the path of the radio waves. Since the beam of radio waves is propagated in a straight line in the tissues, the ratio of the change of absorption of electromagnetic energy in the tissues of the organ being studied to the change in absorption in the remaining tissues, is equal to the ratio of the systolic blood volume flowing through these tissues. Approximate calculation of this value is not difficult and indicates that in the study of such organs as heart, lungs and brain it is not more than 0·05. This means that the error in the contactless method, arising from change in the blood content of tissues surrounding the organ under study, does not exceed 5 per cent.

Experimental verification of the possibility of applying the contactless method to recording processes connected with a change in the quantitative proportions of media of groups I and II has followed two courses. These were experiments on models which, in their electrical characteristics, are the equivalent of the object being studied, and tests on human subjects.

Tests on models had the aim of determining the conditions of maximum sensitivity of the method and of establishing the size of the change in the signal

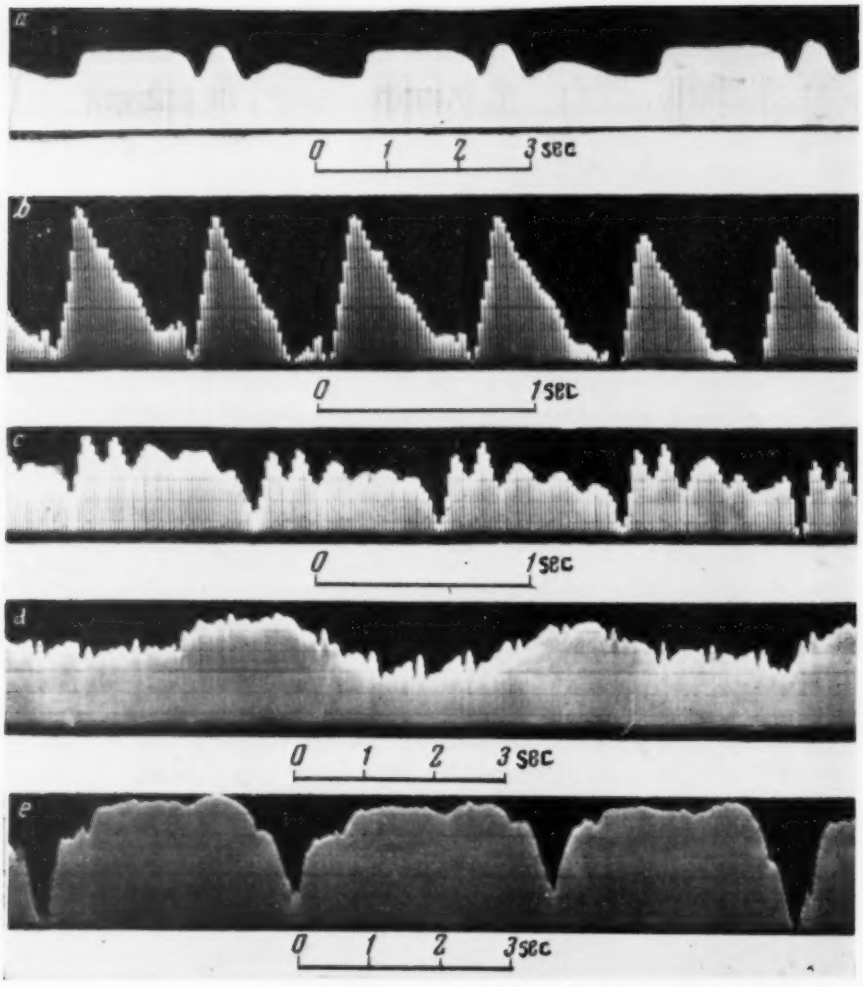

FIG. 6. Examples of use of the contactless method in recording some physiological processes.

in the receiver system when the external dimensions of the experimental object were constant. The model, on which these tests were carried out, consisted of a glass flask with a diameter of 150 mm and a long neck through which was passed a rubber tube, bound at one end and with a rubber bulb at the other. The free space in the flask was filled with 1% NaCl. On compressing the rubber bulb the tube inside the flask expands, as a result of which some of the solution in the flask was displaced into a graduated tube and by the rise in the level of the fluid in this it was possible to estimate the size of the change in the quantitative proportions of air and solution in the flask.

The curves of alteration of radio-wave absorption in the model, produced by periodic compressions of the bulb resulting in a 0·8 per cent change in the quantitative proportions of air and solution in the flask, are shown in Fig. 6(*a*).

These tests enabled the optimum distance between object and antennae to be established, the numerical value of which is given above, and indicated the dependence of change in radio-wave absorption on the internal processes which are in progress in the object under study.

Tests on human subjects were carried out under the same conditions as the model tests. The portion of the body under study was orientated so that the line joining the centres of the antennae (the line of the antennae) passed through the organ under study, and the plane of the transmitting antenna was made parallel to the part of the body on which the projection of the organ of interest was greatest (consideration was also given as to where the amount of "extraneous" tissues, lying in the path of the radio waves, would be least).

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In Fig. 6(*b*, *c*, *d*, and *e*) curves are shown of pulse and respiratory changes of radio-wave absorption in a number of regions of the human body. By positioning the line of the antennae at the level of the *fifth* (Fig. 6*b*) and the *second* (Fig. 6*c*) intercostal space to the left of the breast, the alteration of radio-wave absorption obtained corresponds to the fluctuations of blood content of the heart occurring during inhibition of respiration. The difference in form of the curves (Fig. 6*b* and *c*) indicates that the main radio-wave beam passed through different regions of the heart. These curves were recorded through clothing, whose presence, as the experiment demonstrated, did not affect the sensitivity of the method.

The curve in Fig. 6(*d*) demonstrates the alteration in ventricular volume of the lungs during normal respiration. In this case the group I medium is the air in the lungs, and the group II medium the tissues of the thorax. Increase in radio-wave absorption in Fig. 6(*d*) corresponds to inspiration. In recording this curve the line of the antennae passed through the centre of the chest.

Alteration of the absorption of radio waves when they are propagated in a sagittal direction through the cranium (Fig. 6*e*) corresponds to alteration of the quantitative proportions of blood and spinal fluid, since the total volume of tissues in the brain region remains unchanged. The magnitude of radio-wave absorption is $1{\cdot}0 \pm 2$ per cent of the power passing through the object in the case of recording fluctuations in the blood content of the heart, 3 ± 5 per cent in the case of lung volume and $0{\cdot}3 \pm 0{\cdot}7$ per cent for cranial blood content. The curves presented here are only an illustration of the application of the contactless method in investigating certain functions of an organism and do not exhaust all the possibilities of employment of this method in biological research.

In conclusion, the author expresses his warm thanks to Docent A. I. Naumenko, Candidate of Medical Sciences, for his assistance in organizing the present work.

SUMMARY

(1) The use of u.h.f. currents (in the decimetre waveband) has extended the bounds of existing electrical methods of investigating blood circulation and made it possible to devise a contactless method of studying some of the physiological functions of an organism.

(2) The magnitude of the signal recorded depended on the quantitative proportions of media of groups I and II in the sector of the body through which the radio-wave beam passes.

(3) The greatest sensitivity of the method was found in a sector of the u.h.f. waveband, bounded by wave-lengths of 35 and 80 cm, and when the transmitting-antenna–object and object–receiving-antenna distances were 15 cm and 0·5–1·0 cm respectively.

Translated by C. R. PRINGLE

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THE USE OF ELECTRON MICROSCOPY IN STUDYING THE STRUCTURE OF THE CUTICLE OF NEMATODES*

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THE overwhelming proportion of work on the structure of the cuticle of nematodes has been carried out on ascarids. The existence of ten different layers, in a definite sequence in the cuticle of human, pig and horse ascarids has been established by a number of authors [1–10]. These are: (1) an external cortical layer, (2) an internal cortical layer, (3) a fibril layer, (4) a homogeneous or matrix layer, (5) a band-like layer, (6) an external fibrous layer, (7) a median fibrous layer, (8) an internal fibrous layer, (9) a basal or boundary layer, and (10) a basal or boundary membrane.

It immediately strikes the eye, following enumeration of these layers, that no uniform principle has been taken as a basis for establishing this nomenclature. Thus the first two layers—external and internal cortical layers—and also the last two layers—basal or boundary layer and basal or boundary membrane—obtain their

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names by virtue of their position relative to the other layers, while the remaining layers obtain their names on the principle of their morphological structure. This circumstance can be explained by the fact that the layers were named, not by one author, but by several. At first only four layers were distinguished [11], but subsequent authors added new layers of their own discovery, without changing the names of the old.

To the present time there remains the unresolved question of the presence in the cuticle of ascarids of a system of nutritive canals or slits [8–10], which Goldschmidt [4–5] would on no account recognize. He stated that they were neither passages nor slits, but were in fact thread-like formations, harder than the corresponding layer. At the same time a number of authors [3, 6, 7] assert that the system mentioned above fulfils a nutritive function, understanding this, not as endosmotic nutrition of the helminth, but rather as nutrition of the cuticle as a tissue. Adherents of Goldschmidt ascribe an important structural function to this system.

All the preceding studies of the cuticle were carried out using ordinary histological procedures and stains (haematoxylin, Mallory, etc.). Electron microscopy

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FIG. 1. General view of cuticle (transverse section fixed by Palade's technique, × 1800). (*A*) dense cortical layer; (*B*) diffuse cortical layer; (*C*) membranous layer; (*D*) external layer of longitudinal rods; (*E*) homogeneous layer; (*F*) internal layer of longitudinal rods; (*G*) internal fibrous layer.

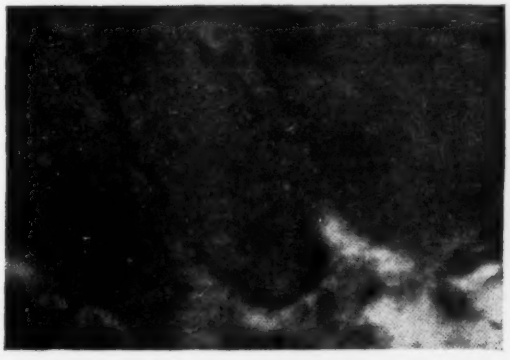

FIG. 2. The internal layer of longitudinal rods (transverse section fixed by Palade's technique, × 16,000).

had not as yet been employed by anyone in the study of cuticle. We considered it would not be without interest to investigate the cuticle of ascarids using the electron microscope, hoping thereby to resolve the controversial questions mentioned above. Besides this we decided to attempt on a factual basis to give more precision to the existing nomenclature of the layers of the cuticle, basing it above all on the characteristics of the layers themselves, mainly in accordance with their structure.

We made a study of more than fifteen adult forms of both sexes of *Ascardia galli* Schrank 1788, a nematode of the ascarid subgroup which lives in the small intestine of a number of birds, mainly fowls.

The structure of the cuticle of *A. galli* has been described only once by Frenzen [12], who discovered six layers in it, being unable to find the internal cortical, fibrillar, median fibrous and basal layers characteristic of other ascarids.

The body length of the helminths studied by us varied from 65–90 mm in females and from 35–60 mm in males. The anterior end in the region of the oesophagous (4–5 mm from the mouth), the middle of the body (in the region of the vulva) and the posterior end (5–6 mm from tip of the body) were studied in each specimen.

The material was fixed by Palade's method in an acetate–veronal buffered 1 per cent solution of osmium tetraoxide at pH 7·3–7·4. After washing and dehydration the specimens were placed in a mixture of butyl and methyl methacrylates (4 : 1). The best results were obtained by fixing pieces of helminth 2–3 mm in length. We had little success in fixing intact helminths, apparently owing to the semipermeable properties of the cuticle. Sections 0·02–0·03 mμ thick were cut on a UM-2 ultramicrotome and were examined without washing of the material in running water using an UEM-100 electron microscope.

Examination of the fine structure of the cuticle of *A. galli* under the electron microscope made it possible, not only to confirm the existence of seven layers which we had detected earlier using an ordinary microscope, but in addition to discover an eighth layer, which is not visible under an ordinary microscope. The newly revealed layer is heavily impregnated with osmium, is fine and homogeneous, and we have named it the membranous layer.

The electron microscope has in addition facilitated a better analysis of the layers in terms of structure (Fig. 1). Thus the outside fine external cortical layer is more heavily osmium-stained than all the other layers, an indication of its great density. The internal cortical layer adjacent to it, which is five to six times broader, on the other hand, has a diffuse structure. In view of the fact that the nomenclature of the layers (external and internal cortical layers) does not reflect their structure, but indicates only their position, we are renaming them, respectively, the dense cortical layer and the diffuse cortical layer.

The membranous layer described above bounds the diffuse cortical layer. Between the membranous and homogeneous layers is the so-called fibrillar layer, which under the ordinary microscope appears as if pierced by transverse fibrils. At low magnification in the electron microscope this layer appears fibrillar. However, at magnifications above $\times$ 2000 it is clearly seen that this layer is composed of closely packed rods, oval in cross-section, passing through the whole length of the body of the helminth (Fig. 2). These rods have a homogeneous structure and, apparently, a distinct membrane, since they are more intensely osmium-stained at the periphery in transverse sections.

The homogeneous or matrix layer is the broadest layer, being $\frac{1}{3}$–$\frac{1}{4}$ the breadth of the cuticle. Under the electron microscope, however, this layer appears far from homogeneous.

According to the date in the literature, in the accepted scheme for ascarids the homogeneous layer is followed by three fibrous layers. Frenzen describes only two fibrous layers—an external and an internal—in the cuticle of *A. galli*.

With the aid of electron microscopy we were able to demonstrate the presence of rods running the whole length of the body in the so-called external fibrous layer, similar to those described by us in the fibrillar layer. Thus the homogeneous layer is bounded on either side by similar layers possessing longitudinal rods. In consequence of this it would be more correct to term the fibrillar and external fibrous layers, external and internal layers of longitudinal rods.

We were not able to observe any fibres in the internal fibrous layer. In the electron microscope it appears to have a diffuse structure.

The basal membrane is relatively fine, but is heavily stained with osmium, and as a consequence is sharply delineated between the internal fibrous layer and the hypodermis.

In examining the cuticle of *A. galli* with the electron microscope we never once were able to observe in it any transverse support structures. If they existed then they would be denser than the layers through which they pass and consequently would be readily observed owing to their more intense osmium staining. On the other hand bands lighter than the corresponding layer, usually not sharply delineated, can be observed in almost all layers of the cuticle.

Thus, using electron microscopy, we find in the cuticle of *A. galli* an additional two layers besides the six described by Frenzen. Therefore the cuticle is represented as consisting of the following layers.

(1) A dense cortical layer (external cortical).
(2) A diffuse cortical layer (internal cortical).
(3) A membranous layer.
(4) An external layer of longitudinal rods (fibrillar layer).
(5) A homogeneous layer.
(6) An internal layer of longitudinal rods (external fibrous layer).
(7) An internal fibrous layer.
(8) A basal membrane.

The use of electron microscopy also enables us to subscribe to the point of view of Toldt concerning the presence of nutritive canals or slits in the cuticle of nematodes, rather than a system of support fibrils as stated by Goldschmidt.

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LETTER TO THE EDITORS

THE CHARACTERISTICS OF ELECTRICAL STIMULATORS*

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MOTOR irritability is determined in clinical and experimental tests from electrical stimulus curves derived by determining the threshold amplitude required as a function of pulse duration. The shape of the curve is determined by (1) the functional state of the tissue, (2) the stimulator characteristics, i.e. the duration, amplitude and shape of the pulse. This is true of both voltage and current pulses, but either the current or the voltage is usually alone recorded. Many commonly used diagnostic instruments (capacitor chronaximeters, accommodometers, some electronic stimulators) record only the voltage; the method is not always suitable, firstly, because the formulae for the excitability curve are usually expressed in current terms (e.g. Nernst's, Lazarev's and Horweg-Weiss's equations); secondly, because living tissues do not follow Ohm's law [1, 2], and thirdly, because the curves expressed in current terms are not the same as those in voltage terms, and so there is no way of comparing results obtained with different types of apparatus [3]. In practice, voltage measurements are still used, however, because no agreement has been reached on dosimetry methods. Voltage measurements have several times been criticized recently [4, 5], and in one instance it is pointed out that if voltage alone is measured neither the amplitude nor the pulse shape at the electrodes is known, even if the source produces rectangular pulses, if the external circuit contains a series resistance of several hundred ohms (a Burginon shunt, etc., may have a resistance of several thousand ohms). This paper deals with a further disadvantage of voltage measurements in isolation.

Allowance has to be made for the skin resistance under clinical conditions, so the voltage within the tissues is not that on the electrodes. The following test was done to demonstrate this difference. The indifferent electrode was fixed to the upper third of the forefinger of an apparently normal subject, and the active electrode to the motor point (counteracting muscle) of the second finger. The electronic stimulator produced approximately square voltage pulses; both current and voltage were recorded. The current was recorded from the voltage drop across a 12·5 Ω series resistance in the external circuit, which had no appreciable effect on the pulse shape. The voltage

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was recorded at the electrodes and also subcutaneously, using a needle electrode inserted near the active electrode. The needle electrode consisted of a hollow needle containing an insulated wire to pick up the voltage. A double-beam oscilloscope (Fig. 1) was used for recording. The threshold voltage was determined as a function of pulse duration at a pulse repetition rate of one per 2 sec. The voltages at points U_1, U_2 and U_3 were then determined at the threshold stimulus level for various

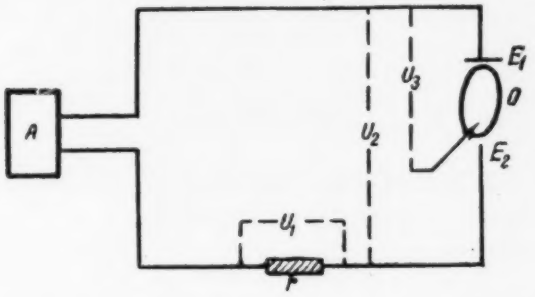

FIG. 1. The electrical circuit. (O) patient; (A) apparatus; (E_1) indifferent electrode; (E_2) active electrode; (r) resistor; (U_1), (U_2), (U_3) voltage used for recording.

pulse durations. The repetition rate was raised to at least 100 c/s for recording photographically from the screen. The subcutaneous and electrode pulses were very different in shape and amplitude, at all amplitudes and pulse durations, as Fig. 2 shows; here the subcutaneous voltage and current pulses (b and c respectively) have been amplified, but the electrode voltage (a) has not. The pulse duration was 0·1 msec. These records show that the electrode voltage does not give the voltage acting on the tissue correctly, and the usual method of recording the voltage cannot therefore be taken as an adequate measure of the stimulus.

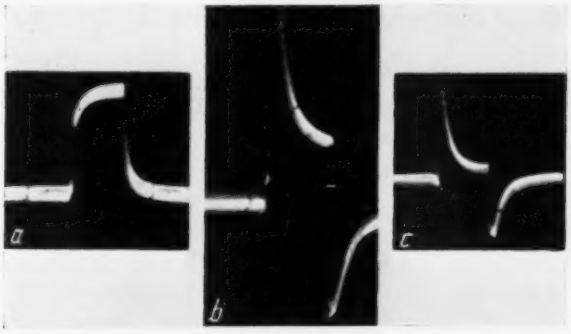

FIG. 2. Pulses in the circuit (see explanation in text).

It is therefore necessary above all else to measure the current with short pulses; otherwise the results are unreliable.

Translated by J. E. S. BRADLEY

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DISCUSSION

THE REQUIREMENTS IN COLORIMETRIC MEASUREMENTS*†

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MY paper [1] on the requirements in colorimetric measurements has caused objections [2] with which, however, I cannot agree. The critics appear to agree with Professor Richter, since they state that "the true meaning of the International Illumination Commission resolutions is that one should not use the 'spectral sensitivity curve' [sic—by which is meant what is usually called the 'visibility curve'] to transfer from three-colour coefficients to match curves". With this I agree, since the 1955 resolutions require that "only complete colorimetric equalities be used", i.e. heterochromatic matches "by brightness only" used in compiling the visibility curve are excluded.

Judd [3] is also quoted as saying: "To understand the meaning of resolution 3, we must remember that no direct measurements of the combination curves had been made up to 1951"; he also states that direct determination of the absolute spectral energy distribution of the instrument was replaced by calculation from the visibility curve. "Resolution 3 expresses the view that any reconsideration of the standard observer of the CIE of 1931 must be based on direct measurements of the combination curves *without distortion* [my italics—N.N.] . . . in relation to flicker photometer data, or using apparatus using brightness matching with appreciable colour differences."

Guild and Wright's work, on which the 1931 standard is based, used visibility curves in transferring from the three-colour coefficients to the combination curves, i.e. results which were not based on complete colorimetric equalities were used. I cannot see how one can deny that Guild and Wright's method has been rejected by the 1955 resolutions. Judd confirms that they replaced the energy parameters of the spectrum by subjective brightness estimates. This situation can, in my opinion, only arise because the problem has not been clearly formulated as a purely objective

* Biofizika **3:** No. 5, 631–632, 1958.

† Reply to Boldyrev *et al.* [2].

physical one, and originates solely from the obscurely formulated subjective concepts against which my paper was directed. At no time have I stated, or found that others have stated, that Guild and Wright did not do their work carefully; I have a profound respect for their experimental skill and for all their scientific work.

As regards Wright's choice of different units for different observers, Wright himself states [4, p. 126] that the readings from the instrument must be multiplied by $1/a_s$, $1/b_s$ and $1/c_s$, or by b_s/a_s and b_s/c_s, where a_s, b_s and c_s are the readings obtained on white light. Since the various observers gave different readings, the result is that physically identical quantities are represented by different numbers after being multiplied. This was precisely the point of my criticism.

Certain of Wright's alterations, designed "to remove the effects of the yellow spot colour", do not affect the discussion, and in fact only confirm that Wright's data give identical figures for different observers who made quite distinct matches, and different figures when they made the same matches. There is no doubt whatever that Wright's data used in compiling the standard had this feature.

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The objections made all relate to some purely illustrative examples in my paper; the main content (particularly the items in the summary) is unaffected, as it dealt with a more rigorous analysis of colorimetric concepts which are not purely elementary (affine character of the colour vector). Such rigour appears unnecessary to many, and I am not convinced that my critics cannot find "nothing that is in principle new" in my paper.

There is no doubt that clarity is essential in propounding biological problems, as the need to reconsider the general substance of the 1931 standard shows. These arguments have been greatly extended and many fresh illustrations given in a more recent article of mine [5].

Translated by J. E. S. Bradley

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* There exists a translation into English of this work.

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STUDY OF NUCLEOPROTEINS: THE STRUCTURAL RELATIONSHIP BETWEEN NUCLEIC ACID AND PROTEIN IN A DNA–α-CHYMOTRYPSIN COMPLEX AND IN NATIVE NUCLEOPROTEINS*

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The most interesting of the considerable number of studies dealing with nucleic acid and nucleoproteins are those which treat of the structural relations of the nucleic acid and the proteins combined in the nucleoprotein molecule [1]. However, few such studies have yet appeared, although the question seems particularly interesting in view of recent information on the changes which occur in the biological activity of proteins as a result of the specific unwinding of the molecules when they combine with nucleic acids [2].

We have attempted to discover some of the properties of nucleoproteins which depend on the quantitative proportions of nucleic acid and proteins combined in the molecule, by investigating model systems and artificial complexes produced from high-polymer deoxyribosenucleic acid (DNA) and individual proteins.

In an earlier work [3], where we studied a DNA–bovine serum albumin complex (DNA–BSA), we showed that artificial and native nucleoproteins had certain physicochemical properties in common: a characteristic lowering of the degree of asymmetry in both, and the emergence of elastic deformation properties. Our study of the quantitative dependence of the intrinsic viscosity and relative relaxation indices of the complexes on their protein content has led us to suggest that there are definite threshold concentrations of the protein in nucleoproteins, which determine the changes in the properties mentioned. This suggestion has not been experimentally confirmed, since the complexes investigated had a relatively high protein content in comparison with native nucleoproteins.

The aim of the present inquiry was to study the DNA–protein interaction under conditions where the relative content of the components approximated to that in the native complex, and to discover the threshold protein-content which would lead to certain qualitative changes in the nucleoproteins. It is particularly worth while finding out what conditions will cause fibrous properties to occur in the structures of nucleoproteins, in view of the information on the isolation of non-fibrillar nucleoproteins which is available in the literature, and the contradictory explanations of the nature of these compounds.

A DNA–α-chymotrypsin complex was taken as the artificial complex for purposes of the investigation. In choosing the protein component for the system we had it in

* Biofizika **3**: No. 6, 633–640, 1958.

mind to study at the same time the influence of nucleic acid on the various forms of biological activity of the enzyme.

The data from this part of the work will form the subject of a separate paper.

METHODS

The DNA preparations were obtained from a freshly frozen bull's pancreas by a modification of the Mirsky–Pollister method [4, 5]. The variation in the method was dictated by the choice as the DNA source of a pancreas exceptionally rich in DNAase and proteolytic enzymes, and consisted in speeding up the processing time as much as possible, and increasing the concentration of sodium citrate (to 0·05 M) used as the DNAase inhibitor. The nucleoprotein was deproteinized by the Sevag method [6]. All the work was performed at 2–4°C. In the DNA preparations the concentration was determined for phosphorus content by the Fiske–Soubarous method; the degree to which the preparations were cleaned of protein was determined by the N/P ratio (Conway determination of nitrogen); and the molecular weight by viscometry [7]. An Oswald viscometer with capillary diameter of 0·6 mm and length of 10 cm was used to measure the viscosity of the DNA dissolved in 0·2 M NaCl, at $25 \pm 0{\cdot}01$°C. The following were the indices for the three DNA preparations used in the work: N/P = 1·89, 1·84, 1·79; M = $1{\cdot}6 \times 10^6$, $3{\cdot}5 \times 10^6$ and $3{\cdot}3 \times 10^6$.

Under the conditions described above, the viscosity of the deoxyribosenucleoprotein (DNP) dissolved in 0·5 M NaCl solution, was equal to that of a solution of 0·004% DNA.

The protein and nucleic acid content in the complex was calculated from the nitrogen–phosphorus ratio. The degree of relaxation of the DNA fibres was determined by a method worked out by one of the present authors [8]. The DNA fibres were produced in the same media and under the same conditions as the artificial complex.

Crystalline α-chymotrypsin was obtained by the Kunitz and Northrop method [9]. After dialysis the preparations were subjected to lyophilic drying or kept cold in a 0·01 N H_2SO_4 solution. Under these conditions the activity of the chymotrypsin was preserved for several months. The DNA–α-chymotrypsin complexes were produced in a citrate buffer with an ionic strength of 0·14. The DNA which was to be added to the protein solution in the buffer was first precipitated from two volumes of alcohol and introduced into the solution in gel form. The complexes were subsequently separated and purified according to a method described in an earlier work [3]. The work was carried out in the cold.

EXPERIMENTAL CONDITIONS FOR THE FORMATION OF DNA–α-CHYMOTRYPSIN COMPLEXES

Examination of the optimum conditions of protein concentration and pH of the medium for forming the complex (Table 1) showed that the complex could be produced under various pH conditions, depending on whether the protein was in monomer or dimer form. It is known that chymotrypsin can be subjected to a process of inverse dimerization [10, 11], and that under certain conditions of concentration and pH of the medium it exists in solution in the monomer form, the dimer form, or a mixture of molecules in both states.

As can be seen from the data given above, in concentrations where the chymotrypsin is in solution in dimer form or as a mixture of both states (10 mg/ml), it

behaves as a basic protein and forms insoluble complexes over a wide range of pH of the medium, from four to seven inclusive; whereas in concentrations where the chymotrypsin is exclusively in the monomer state (1 mg/ml) it behaves as an acid protein and forms insoluble complexes with nucleic acid at a pH not exceeding 5·8. These somewhat unexpected results may correspond to the known data [12] on the different isoelectric points of this protein (8·1–8·3 and 6·0–6·3) which have been found when different methods of calculation have been used.

TABLE 1. YIELD OF DNA COMPLEX WITH MONOMERIC AND DIMERIC α-CHYMOTRIPSIN AS A FUNCTION OF THE pH OF THE MEDIUM

pH	Concentration of α-chymotrypsin in medium (mg/ml)	DNA (mg)	Complex yield, calculated by N (mg)
	Dimer		
4·5	10	1·72	7·56
5·3	10	1·72	7·95
5·5	10	1·72	8·36
5·75	10	1·72	7·43
6·1	10	1·72	7·06
6·6	10	1·72	6·94
7·0	10	1·72	6·31
	Monomer		
4·5	1	0·22	1·24
5·3	1	0·22	0·81
5·5	1	0·22	0·37
5·8	1	0·22	Traces
6·4	1	0·22	0

From the first part of Table 1 it can be seen that the yield of complex diminishes as the pH of the medium increases. The explanation may be that under these conditions there is a constant lowering of the degree of dimerization of the chymotrypsin in the solution (this agrees with data given in the literature [10]), and a consequent lowering of the dimer concentration in the medium. The observed regularity may serve as indirect proof that under these conditions the DNA reacts with the chymotrypsin dimer.

The complexes produced with monomeric chymotrypsin showed the characteristic nucleoprotein lowering of viscosity and were precipitated from physiological saline solution. Better results were obtained with a pH of 4·5. These conditions were also adopted for the subsequent study of the properties of this complex.

QUANTITATIVE CORRELATIONS OF PROTEIN AND DNA IN THE COMPLEX

As stated in the previous paper, complexes of different compositions can be obtained by varying the relative quantities of protein and DNA entering into the interaction: the higher the initial protein–DNA ratio, the more protein combines with

the nucleic acid in the complex. In order to find out the minimum and maximum quantities of protein capable of combining with a molecule of DNA, we made a comparative study of the composition of complexes produced from different initial ratios of the components, taking DNA–protein ratios from 1 : 0·1 to 1 : 600. Figure 1 shows the results, compared with the results obtained when the DNA–BSA complex was studied [3].

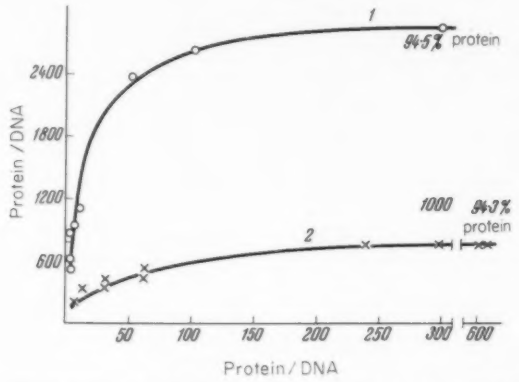

Fig. 1. Dependence of quantity of protein in complex on initial ratios of the components. (1) DNA–α-chymotrypsin complex; (2) DNA–BSA complex. Abscissa: initial values of protein–DNA ratio. Ordinate: quantity of protein moles to 1 mole of DNA.

As the graph shows, we succeeded in achieving near-saturation of protein in the DNA, the maximum quantity of protein (taking the molecular weight of the monomer as 21,600 [9]) being 2800–2900 moles to one of DNA, or an average of about 94·5 per cent in terms of weight. The corresponding magnitude for albumin was 1000–1100 moles or an average of 94·3 per cent. (If the maximum quantity of chymotrypsin which combines with the DNA is converted, according to the molecular weights into moles of albumin, we obtain a magnitude near to that obtained for the DNA–BSA complex.)

Comparison of the findings for the two types of complex shows that the quantity of protein capable of combining with a molecule of DNA depends on the molecular weight of the protein, or in other words on the proportional magnitudes of the areas of the molecules in reaction; but the quantity of proteins of different types which combines with a molecule of DNA is nearly the same, in respect of weight. We find confirmation of this feature again if we compare (in similar experimental conditions) the quantities of protein entering into interaction with DNA preparations of different degrees of polymerization. The quantity of protein (in moles) capable of combining with the nucleic acid depends on the molecular weight of the nucleic acid, although in terms of weight different DNA preparations attach to themselves practically the same quantities of protein (Table 2).

Rather different results were obtained when we compared the quantities of monomeric and dimeric chymotrypsin which attach to DNA. In both cases, the quantity of protein attaching to a molecule of DNA was the same in terms of molarity, whereas in terms of weight the dimer form combined to roughly twice the quantity of the monomer form.

TABLE 2. DEPENDENCE OF THE QUANTITY OF PROTEIN COMBINING IN DNA–α-CHYMOTRYPSIN COMPLEX ON THE MOLECULAR WEIGHT OF DNA

DNA: protein (initial values)	Composition of protein–DNA complex			
	In moles		In weight units	
1: 0·25	223*	533†	3·1*	3·3†
1: 0·5	289	615	3·9	3·8
1: 5	385	922	5·2	5·7
1: 10	503	1070	6·8	6·4

* Throughout column DNA = $1{\cdot}6 \times 10^6$.
† Throughout column DNA = $3{\cdot}5 \times 10^6$.

TABLE 3. EFFECT OF DIMERIZATION OF α-CHYMOTRYPSIN ON ITS CONTENT IN THE DNA–α-CHYMOTRYPSIN COMPLEX

DNA: protein			
Moles		Weight units	
Monomer*	Dimer†	Monomer*	Dimer†
316	307	4·2	8·2
355	307	4·8	8·2
304	269	4·1	7·2

* Throughout column molecular weight = 21600.
† Throughout column molecular weight = 43000.

We obtained the same sort of results when we studied the quantities of native and denatured serum albumin combining with a molecule of DNA [3]. This may in some sense bear out our earlier hypothesis on the formation of albumin dimers during denaturing.

SOME PHYSICO-CHEMICAL PROPERTIES OF DNA–α-CHYMOTRYPSIN COMPLEXES

Viscosity

Certain physico-chemical properties of the complexes were studied: intrinsic viscosity, and capacity for relaxation. From the data given (Fig. 2) it can be seen the viscosity of the complexes has diminished roughly twofold by comparison with the original nucleic acid viscosity. This fully accords with our earlier published results, as well as with other data to be found in the literature in connection with the study of other objects.

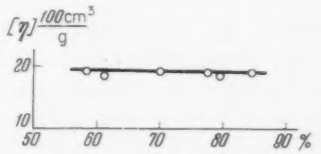

FIG. 2. Dependence of intrinsic viscosity of DNA–α-chymotrypsin complex on quantity of protein contained in it. Abscissa: percentage of protein in DNP.

In the present experiment, as in the previous one, we did not succeed in obtaining viscosity values intermediate between that of the original DNA and that of the complex, apparently because the quantity of protein in the latter was considerably greater than the quantity necessary to obtain the maximum effect of viscosity reduction, which for various structural reasons is 20–30 per cent [1].

Contractile properties of a DNA–α-chymotrypsin complex

The contractile properties of nucleoproteins are, as previous investigations have shown, functions of their protein content and of the concentration of the complex in solution. To discover the quantitative dependence of the degree of relaxation on the protein content, concentrations of the complex were measured to determine the optimum, which was found to be 6 mg/ml. Fig. 3 shows the characteristic contractile

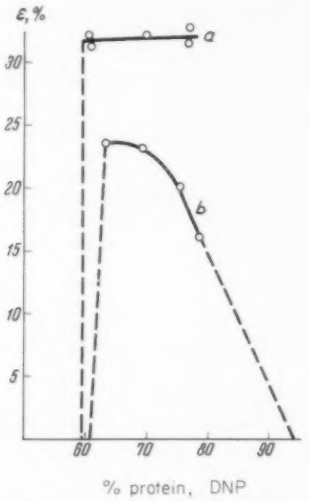

Fig. 3. Dependence of relative relaxation of native nucleoproteins and artificial DNA–α-chymotrypsin complexes on the quantity of protein contained in them. (*a*) DNP from pancreas. (*b*) DNA–α-chymotrypsin complex. Abscissa: percentage of protein in DNP.

properties of the complex, expressed in percentages of relative relaxation as a function of the protein content. As the graph shows, the DNA–α-chymotrypsin complex displays contractile properties, and its capacity to form relaxed fibres starts when a definite level of protein content is reached (roughly 61–63 per cent). This agrees with our own earlier results [4] and also with the data given in the literature [13, 14]. The elasticity of the complex is at its maximum in a definite range of protein contents (up to 70 per cent), and remains roughly constant; but if the quantity of protein increases the elasticity diminishes, apparently as a result of the increased number of intra- and inter-molecular bonds, which make the molecule less flexible.

If the straight line corresponding to the relaxation of the complex is extrapolated to the abscissa axis, we obtain the value corresponding to the zero degree of relaxation of the nucleoprotein; that is, complete screening of the DNA bonds around which free revolution and, naturally, multiplication of intermolecular joints, is possible. We may regard the DNA molecule as completely saturated with protein when this

happens, the protein quantity being about 94 per cent. This magnitude is in good agreement with the maximum quantity of protein (94·5 per cent) obtained from other experimental findings (see above) as able to combine with a DNA molecule.

The value for the threshold concentration of protein which will cause a nucleoprotein to undergo qualitative conversion into an elastic fibrous structure is of major interest. This transition is preceded by an intensely hydrated gelatinous condition of the nucleoprotein, which we noticed distinctly in all the experiments. The gel was not precipitated as a result of dilution, or was precipitated in the form of a flaky deposit which did not give fibrillar threads. These properties were observed in nucleoproteins which had a protein content of less than 60 per cent. We obtained similar results with the DNA–BSA complex.

It was seen that the observations described above could have value in connection with existing data given in the literature concerning the isolation of non-fibrous nucleoproteins, of which the formation mechanism has not been explained, from the nuclei of a number of organs. It was necessary, however, to find out how far the regularities which had been observed in the artificial complex could be extended to native nucleoproteins. Experiments were therefore carried out in which native nucleoproteins obtained from the pancreas and the liver of a bull were gradually deproteinized. The deproteinization was performed by agitating the nucleoprotein solution in 1 M NaCl for a short time in an equal volume of chloroform and N-butane (3 : 1). After each deproteinization the N/P ratio was determined, and also the degree of relaxation of the nucleoprotein in physiological saline solution. Tests performed at three different concentrations gave similar results (Fig. 3). The same regularity was observed for native nucleoproteins. Solutions of the nucleoproteins in 1 M NaCl gave fibrous deposits when diluted and fibres which relaxed in physiological saline when the protein content was within the range 60–63 per cent. On further deproteinization, the nucleoprotein was precipitated in the form of a non-fibrous deposit and no longer gave relaxed fibres. This condition occurred when the protein content was 58–60 per cent. After this, further removal of the protein yielded a preparation soluble in physiological saline. Similar results were obtained with a nucleoprotein preparation which was subjected to 72 hr dialysis against 1 M NaCl. In this case a nucleoprotein was immediately obtained which on dilution gave a non-fibrous deposit and was converted into a soluble state at the very first deproteinization.

The results obtained in this way showed that some sort of regularities are common to artificial high-polymer DNA complexes with various proteins and to native nucleoproteins. It seems that, in both kinds of complex, fibrous properties occur as a result of identifical regularities, based on raising the degree of dehydration of the DNA molecule and increasing the forces of aggregation which constitute the conditions for the formation of supermolecular structures.

DISCUSSION

Comparison of the maximum quantities of the two different proteins, α-chymotrypsin and BSA, which enter into a complex with DNA and have different molecular weights and a different quantity of basic groups, shows that the molar proportions of DNA and protein entering into the complex depend on the molecular weights of the DNA and the proteins; while the weight ratio of the DNA and the protein remains

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constant for the different proteins. This indicates that not only do the salt and other bonds involved play a great part in forming the DNP, but also the relative magnitudes of the areas of the reaction molecules.

Our investigations of the characteristics of the physico-chemical properties of the complexes showed that the nucleoproteins formed by the interaction of nucleic acid with a protein are qualitatively new compounds, compared with the components of which they are made, and possess a number of qualitatively new features: high elasticity, a lower molecular asymmetry than DNA, and a solubility which is distinct from that of DNA. The appearance of the new properties depends, within definite limits, on the quantitative proportions of nucleic acid and protein entering into the composition of the new compounds.

The measurements which we made taking DNA–α-chymotrypsin as the example for artificial complexes and DNA–BSA as the example for native nucleoproteins, showed closely similar limits for the protein content which would cause relaxation properties to appear, in both the artificial and the native complexes (roughly 58–60 per cent; N/P = 3·8–4·1).

The qualitative change in the nucleoprotein which is accompanied by the emergence of certain structural–mechanical properties (ability to form fibrous structures and relaxed fibres in a given medium) is preceded by another condition, characterized by a protein content of the order of 58 per cent or lower and by a greater degree of hydration. Nucleoproteins in this state give non-fibrous deposits, which do not form fibres on dilution. Again, we obtained this qualitative change in experiments both with the model systems and with the native nucleoproteins. It appears to be characterized by the presence of definite intermolecular forces, which cause the formation of gels but are insufficient to form strictly orientated fibrous structures. In connection with the data we have obtained, it is worth noting the information given by a number of other investigators [14–16] on the isolation of non-fibrous DNP from the nuclei of rats' livers; these products are distinguished by higher solubility and on dilution yield flaky deposits instead of a fibrous mass. Study of the composition of amino acids and nitrous bases has shown that the fibrous and non-fibrous forms are chemically identical, and that the protein content in the latter is 58–59 per cent. Preparations made from different sources (calf's thymus, rat's liver and spleen) do not differ in their properties.

It is difficult to compare the data given in these works with the results of our own experiments without sufficient knowledge of the details of the investigation. It may be assumed, however, with a high degree of probability, that the non-fibrous preparations were obtained because of the methods the authors used in order to isolate them. These methods entailed prolonged extraction and dialysis, during which partial deproteinization of the DNA was observed; this resulted in a lowering of the protein content required for the formation of a fibrous structure of the preparation.

Our findings show that a slight alteration of the protein content (of the order of 1–2 per cent) is sufficient to cause a qualitative disturbance of the fibrous properties of the DNP. This gives grounds for assuming that such alterations play a definite part also in the living medium of the organism, and that an interchange of fibrous and non-fibrous states of the nucleoprotein plays a definite part in the processes of its directed exchange.

SUMMARY

(1) DNA complexes were obtained with monomeric and dimeric α-chymotrypsin.

(2) It was found that the maximum (by weight) quantity of α-chymotrypsin which could enter into a complex with DNA corresponded to the quantity of albumin in a DNA–BSA complex.

(3) The relationship between certain physico-chemical properties of the complexes and their protein content was studied.

(4) A threshold protein content was established in both artificial complexes and native nucleoproteins, determining the fibrous properties of the structures.

(5) A suggestion has been made concerning the nature of the non-fibrous nucleoproteins described in the literature.

Translated by KENNETH SYERS

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* There is an English translation of this work.

THE INFLUENCE OF ULTRASONIC WAVES ON THE ELECTROKINETIC POTENTIAL OF CELLS*

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IT has been previously suggested that under the influence of ultrasonic waves albuminous molecular complexes combined with the surface layers of cells and tissues undergo certain definite physico-chemical changes [1–4]. This has been borne out by data which show that those enzymes, which are primarily located on the surface

* Biofizika **3:** No. 6, 641–646, 1958.

of cells or cell structures, are activated by ultrasonic oscillations (invertase, cholinesterase, ectoapyrase of nuclear erythrocytes).

The present paper gives data which directly show that physico-chemical changes are observed in cell membranes as a result of the action of ultrasonic waves. The changes occur when the ultrasonic waves are comparatively weak in intensity, so that the possibility of cavitation is obviously excluded. The electrokinetic potential (ζ-potential) of cells has been studied. As is known, the ζ-potential is a magnitude which characterizes the physico-chemical state of a surface, its isoelectric point, the thickness of the electric double layer, and so forth.

The results of the investigation have enabled us to assess not only the disturbances caused by ultrasonic waves in the physical condition of cell membranes, but also the forces which initiate these disturbances and which are directly bound up with the propagation of ultrasonic oscillations (pressure variation, displacement, and acceleration of particles).

The physico-chemical changes observable in cell surfaces take place according to whether the cells are in the process of ultrasonic stimulation in the acceleration antinode (pressure node) or acceleration node (pressure antinode).

EXPERIMENTAL

To measure the electrophoretic mobility of the cells we used our own modification of an Abramson–Moyer chamber [5, 6]. The subject of investigation was the erythrocytes of a rat, which are distinguished by having a stable ζ-potential of considerable magnitude.

Erythrocytes isolated from fresh rat's blood were washed three times in physiological saline solution, and then suspended in a 1/15 M phosphate buffer (pH 7·4) diluted with 1% NaCl in proportions of 1 : 9. To determine the isoelectric point of the erythrocytes we studied their electrophoretic mobility in buffer solutions prepared by the Clark method and diluted with 1% NaCl solution in the proportions indicated above.

The ionic strength μ of all the solutions we examined thus remained equal to 0·17. This was necessary in order that we might compare the results obtained by measuring the electrokinetic potential. The voltage supply came from a rectifier through an electronic current stabilizer to non-polarized copper electrodes. The experiments were carried out at a potential gradient of 5 V/cm. The microscope observations were made through the upper wall of the chamber by means of an opaque illuminator. The objects were illuminated through a thermal filter. The measurement results were expressed in terms of the electrophoretic mobility of the cells in (μ/sec)/(V/cm) measured at the mid-depth of the chamber.

The erythrocytes were subjected to ultrasound in the chamber itself. A piezoquartz plate constituted the source of the ultrasonic waves. These were fed to the chamber through a glass tube filled with water. The lower end of this tube was covered with a polythene film, which gave practically complete admission to the ultrasonic energy striking it. Contact between this tube and the source of ultrasound was effected through the layer of water bordering the upper surface of the quartz plate (Fig. 1*a*). The frequency of the ultrasonic waves used was 400 and 800 kc/s.

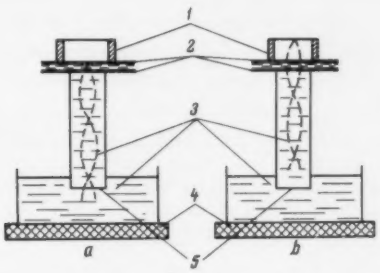

FIG. 1. Scheme for subjecting cells to ultrasonic waves in an electrophoresis chamber: (a) in antinode of acceleration; (b) in antinode of pressure. 1—glass ring; 2—electrophoretic chamber; 3—water; 4—piezoquartz plate; 5—polythene film.

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We used the same chamber for all the experimental work, keeping it at a constant distance from the crystal. The intensity of the ultrasonic waves was gauged from the thermal effect (colorimetric method). The thermal effect of the ultrasound was determined by means of an instrument recently described by Wiederhielm [7].

In order that we might be able to find out the mechanism by which the low-intensity ultrasonic waves affected the erythrocytes, they were subjected to the ultrasound separately in the acceleration node and the acceleration antinode. For this purpose conditions were arranged such that the chamber with its contents was situated either in the antinode of acceleration or in the node of acceleration of the stationary wave.

Bearing in mind the small thickness of the upper and lower walls of the chamber, which consisted of quite thin glass covers (0·03 mm), we assumed that the reflection of the ultrasonic energy from the upper wall was produced in the same way as from a water-air division boundary. If this was so, then as theoretical calculation shows [8], the slight phase displacement in our tests would not exceed 2°, and would therefore play no noticeable part.

With some approximation, we may take it that an antinode of acceleration or a node of pressure occurred in the upper wall of the chamber (Fig. 1*a*). The phase would be approximately the same through the chamber, since the depth of the chamber (0·1 mm) was considerably less than the wave-lengths, which were roughly 4 and 2 mm in the aqueous medium with the ultrasonic frequencies used.

In order that the chamber should be in the node of acceleration, a glass ring of height a quarter or three-quarters of the wave-length used in the water was attached to the upper wall. This ring was filled with water. Under these conditions, as calculation shows, there will be an antinode of pressure or a node of acceleration in the upper wall of the chamber (Fig. 1*b*).

In order to establish a correct pattern of stationary waves in the system, we used a method well known in ultrasonic interferometry. This is based on the principle that when resonance occurs in the system, the strength of the current passing through quartz increases ("reaction" to quartz). When quartz is excited in water it is easier to record the sharp minima of current which can be observed under conditions furthest away from resonance, than the dense maxima which occur in connexion with

resonance [9]. Therefore in order to apply the ultrasound to the objects of investigation in the antinode of acceleration, we set the chamber at a distance from the quartz corresponding to the mean position between neighbouring current minima. For applying the ultrasound in the node of acceleration, we found a position for the chamber corresponding to the current minimum passing through the quartz, and then filled with water the glass ring attached to the upper wall of the chamber.

Table 1. Dependence of electrophoretic mobility on the pH of the medium, as found for the erythrocytes of a rat at different times after their separation from freshly obtained blood

pH values of measurement buffer	Electrophoretic mobility of erythrocytes $\left(\frac{\mu/\text{sec}}{\text{V/cm}}\right)$ after keeping them in phosphate buffer (1/15 M, pH = 7·4)			
	30 min	60 min	120 min	360 min
10·28	1·56	1·64	—	1·42
8·38	1·42	1·50	—	1·38
7·70	1·42	1·50	1·48	1·36
7·38	1·43	1·44	1·42	1·38
5·60	0·97	0·62	0·43	0·60
4·66	0·48	0·70	0·00	0·01
3·82	0·17	0·00	−0·10	−0·30
3·24	−0·05	−0·18	−0·40	−0·52
2·92	−0·19	−0·21	—	—

We may note that, according to our measurements, the electrophoretic mobility of the erythrocytes 30 min after they had been placed in the buffer solution (1/15 M phosphate buffer, pH 7·4) was 1·45 (μ/sec)/(V/cm); this coincides with the data given in the literature [10]. (In our account we give magnitudes of the electrophoretic mobility which completely correspond to the ζ-potential values.) It is known, however, that the value of the ζ-potential or of the electrophoretic mobility of erythrocytes spontaneously falls about 2 hr after they have been withdrawn from the organism. There is then a displacement of the isoelectric point of the surface layer of the erythrocyte in the alkaline direction [10, 11], as was confirmed by our experiments. As can be seen from Table 1, the isoelectric point of the erythrocyte, that is to say, the smallest charge on its surface, was observed when the pH was 3·24, if the electrophoretic mobility was measured 30 min after the erythrocytes had been separated from the freshly obtained rat's blood. When the electrophoretic mobility was measured 2 hr after separation of the erythrocytes, the isoelectric point was found to occur at pH 4·66.

It must be emphasized that the ζ-potential or electrophoretic mobility of the erythrocytes 2 hr after they have been separated from fresh blood is under ordinary conditions a stable magnitude. Accordingly, in studying the effect of ultrasonic waves on the electrophoretic mobility of the erythrocytes, we carried out two series of investigations. The first series comprised tests with erythrocytes in the stage of spontaneous diminution of the ζ-potential, and the second series tests with erythrocytes with a stable ζ-potential.

The results of the first series of measurements of the electrophoretic mobility are set out in Table 2. As this shows, the electrophoretic mobility of the

erythrocytes, measured 30 min after their separation from fresh blood, distinctly diminishes under the influence of ultrasonic waves.

The application of the ultrasound and the actual measurements took a few minutes in all. Control measurements (without application of ultrasound) taken over the same period failed to show any change in the electrophoretic mobility of the erythrocytes studied.

TABLE 2. INFLUENCE OF ULTRASOUND ON ELECTROPHORETIC MOBILITY AND ISOELECTRIC POINT OF THE SURFACE LAYER OF FRESH ERYTHROCYTES KEPT FOR 30 MIN IN A PHOSPHATE BUFFER (pH = 7·4)

pH of solution on which measurements were made	Electrophoretic mobility of erythrocytes $\left(\frac{\mu/\text{sec}}{\text{V/cm}}\right)$	
	Before application of ultrasound	Ultrasound for 3 min (intensity 0·02 W/cm²)
7·40	1·45	1·26
5·71	1·13	0·94
4·80	0·80	0·60
4·52	0·40	0·21
4·00	0·39	—0·04
3·70	0·00	—0·11
3·38	—0·12	—0·20
3·02	—0·20	—

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The diminution in the electrophoretic mobility of erythrocytes under the influence of ultrasonic waves depended on the intensity of this physical agent (Table 3). The greater the intensity of the ultrasound, the more rapidly did the isoelectric point of the erythrocyte shift towards the alkaline. We observed a rapid lowering of the electrophoretic mobility of this kind directly after the objects had been subjected to ultrasonic influence of an intensity of 0·02 and 1 W/cm². In this case another phenomenon, described in an earlier work [12] was also observed: the erythrocytes began to revolve about their axis in the field of the ultrasonic waves. The revolution stopped almost instantaneously when the ultrasonic wave generator was switched off, and was resumed as soon as it was cut in again.

The results of the first series of tests, as set out above, were obtained under conditions in which the chamber with the erythrocytes was placed in the antinode of acceleration of the ultrasonic wave during the operation of the ultrasound. As a rule we did not find that the ζ-potential of the erythrocytes was affected when they were in the node of acceleration or antinode of pressure during the operation of the ultrasound (Table 3).

This rule was found to be independent of the frequency used in the test (400 or 800 kc/s).

A similar regularity was again found in the second series of investigations, when the influence of the ultrasonic waves on erythrocytes with a stable ζ-potential (under ordinary conditions) was studied. The only difference here was that the shift of the isoelectric point of the surface layer of the erythrocytes under the influence of the ultrasonic waves (in the antinode of acceleration) was of the opposite nature. To investigate this we used erythrocytes which had previously been left in a buffer

Table 3. Effect of ultrasonic waves on electrophoretic mobility of erythrocytes as a function of the experimental conditions. (The erythrocytes were placed in a 1/15 phosphate buffer, pH 7·4, for 30 min)

Time of application of ultrasound	Electrophoretic mobility of erythrocytes $\left(\frac{\mu/sec}{V/cm}\right)$ during application of ultrasound			
	In acceleration antinode		In pressure antinode	
	0·02 W/cm²	1 W/cm²	0·02 W/cm²	1 W/cm²
3 sec	1·42	1·40	1·44	1·42
10 sec	1·42	1·39	1·44	1·40
30 sec	1·38	1·36	1·43	1·40
1 min	1·36	1·33	1·42	1·38
2 min	1·34	1·32	1·43	1·41
3 min	1·34	1·28	1·42	1·42

Note: Control (without application of ultrasound) 1·44.

solution for 4 hr after they were taken from the fresh blood. In these cases the fall in ζ-potential could be observed only a short time after the action of the ultrasonic waves. Two min after the action of the ultrasonic waves the electrophoretic mobility of the cells reverted to its previous magnitudes, that is, to the magnitudes characteristic for intact erythrocytes (Table 4).

Table 4. Duration of after-effect when erythrocytes were subjected to ultrasound for 3 min at an intensity of 0·02 W/cm²

Time (min) during which erythrocytes remained in 1/15 M phosphate buffer, pH 7·4, before application of ultrasound	Control (without ultrasound)	Electrophoretic mobility of erythrocytes $\left(\frac{\mu/sec}{V/cm}\right)$ at various times after action of ultrasound				
		15 sec	1 min	2 min	10 min	30 min
30	1·43	1·34	1·32	1·34	1·32	1·30
360	1·38	1·32	1·32	1·37	1·38	1·36

DISCUSSION

The electrokinetic potential of rats' erythrocytes was observed to diminish when the intensity of the ultrasound (0·02 W/cm²) was such that no cavitation occurred. It was found to occur when the erythrocytes were subjected to the effect of ultrasound in the antinode of acceleration, but not when this was done in the node of acceleration. It is noteworthy that the lowering of electrokinetic potential had the opposite character if erythrocytes with a stable ζ-potential (under ordinary conditions) were subjected to the effect of ultrasound in the antinode of acceleration.

These variations could apparently be observed only because we used a very sensitive method of investigation to determine the electrokinetic potential. It is known that even quite small changes in the concentration of electrolytes in the medium lead to a considerable change in the ζ-potential of colloidal particles. At the same time, it

is known [13] that in true solutions of the electrolytes, under the influence of an acceleration of the particles in the field of ultrasonic waves, periodic oscillations of the ion concentrations occur, along with a variable difference in the potentials. The differences in the potentials are caused by the fact that ions with different masses, acquiring different accelerations in a field of ultrasonic waves, are at a definite distance from one another. This effect quickly disappears when the ultrasonic field is removed. With colloidal solutions or thin suspensions, however (under stationary wave conditions), micelles or suspended solid particles, concentrating in nodes of acceleration or displacement, remain there even after being subjected to ultrasound for many seconds [14, 15]. Some authorities have reached this conclusion on the basis of investigating the characteristics of the diffraction of light as a result of ultrasound.

We are inclined to regard in this light the changes in the electrokinetic potential of erythrocytes in an antinode of displacement or acceleration, which we observed. We may take it that, in a field of ultrasonic waves, not only does the ion envelope around an erythrocyte disappear in an antinode of acceleration, but the colloid particles which enter into the composition of the surface layer of the erythrocyte are displaced as well. As a result, there may occur other conditions of dissociation of the acidic or basic groups of protein on the surface of a cell, which cause the isoelectric point of the erythrocytes to shift.

The investigations we have made open up new possibilities for interpreting the mechanism by which ultrasonic waves act on the functional characteristics of living membranes (enzyme activity, diffusion phenomena, penetration processes and so on).

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SUMMARY

(1) Under the influence of low-intensity ultrasonic waves, the electrokinetic potential of erythrocytes falls.

(2) When erythrocytes are subjected to the effect of ultrasound before their ζ-potential has attained a constant magnitude, the fall in value of the ζ-potential is speeded up. An electrokinetic potential which has already been established (after the erythrocytes have been kept for some time) also falls as a result of the effect of ultrasonic waves. In this case the effect continues for some time after the operation of the ultrasonic waves.

(3) The diminution in the ζ-potential of erythrocytes occurred in the antinode of acceleration but did not occur in the antinode of pressure of a stationary ultrasonic wave.

(4) We have discussed a possible mechanism which may account for the physicochemical changes which take place on the surface of erythrocytes during their subjection to the influence of ultrasonic waves.

Translated by KENNETH SYERS

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THE CAUSES OF SOUNDS PRODUCED IN ARTERIES WHEN BLOOD-PRESSURE CUFFS ARE APPLIED*

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(*Received* 15 *April* 1957)

THE acoustic method of studying arterial pressure first proposed by Korotkovskii in 1905 is now used throughout the world, though the causes of the sounds have not been explained. We have shown that the sound in an artery arises from the systolic increase in blood flow rate, i.e. from the rate at the instant when a pulse propagates along the arteries. This increase is much greater when a cuff is applied, which causes water-hammer sounds in the arteries [1–5]. This explanation may appear paradoxical; it is difficult to see why the energy of the pulse wave is insufficient to cause a sound when no cuff is applied, but sufficient when a cuff is applied. This paper presents an analysis of this latter fact.

The arteries have elastic walls, so the contracting heart does not have to displace all the blood in the arteries at once. The systolic blood begins to enter the central arteries, which expand to accommodate the extra blood; the pressure meanwhile remains minimal (diastolic) elsewhere. The blood flow between the regions of maximum and minimum pressure increases; this increased flow propagates along the arteries in company with the pressure wave and causes the arteries to distend. This is the pulse wave, which propagates at a rate dependent on the arterial elasticity, and which may have a speed of several metres per second. We now examine the conditions which determine the speed of the blood stream at any point when the pulse wave reaches it.

Let A and B be two cross-sections of the artery a short distance l apart (Fig. 1). The pulse wave is of speed λ and takes a time t_1 to pass from A to B; $t_1 = l/\lambda$. A pressure difference arises between A and B when the wave has just reached A; an element of blood of mass m at point A is given an acceleration a_1 proportional to the force p applied. The velocity U_1 which this blood attains on moving between A and B is $U_1 = a_1 t_1$. We then have

$$U_1 = (p/m)\,(l/\lambda). \tag{1}$$

* Biofizika **3:** No. 6, 648–656, 1958.

The velocity is thus proportional to p and inversely proportional to λ; the result also follows from Zhukovskii's classical formula [5] $\Delta P = \beta\lambda\Delta W$, where ΔP is the pressure rise, λ is the wave velocity, ΔW is the increase in the mean flow rate and β is the density.

If the arterial pressure at systole rose instantaneously from P_{min} to P_{max} the pressures at A and B would have these values, and p would be $P_{max}-_{min}$, i.e. the pulse pressure P. The rise is not instantaneous, though; a time T is required. The

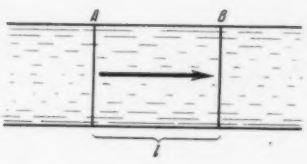

FIG. 1.

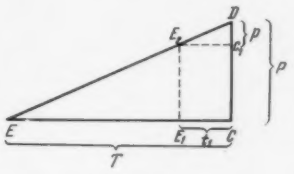

FIG. 2.

rate at which the blood is ejected is proportional to $1/T$. The pressure can rise by an amount p, less than $P = P_{max}-P_{min}$, during the time t_1. Figure 2 is the pressure-time curve (CD vs. EC), assuming uniform rates; CED is then a right-angled triangle. The time t_1 is represented by $E_1C = E_2C_1$; the pressure rise in this time is C_1D. Triangles CED and C_1E_2D are similar, so $E_2C_1/EC = DC_1/DC$, or $t_1/T = p/P$, so $p = t_1P/T$; p is proportional to P and to $1/T$. Equation (1) then gives $U_1 = (Pt_1/Tm)(l/\lambda)$; but $t_1 = l/\lambda$, so $U_1 = Pl^2/Tm\lambda^2$. We now put $l^2/m = n$; we then have

$$U_1 = nP/T\lambda^2. \tag{2}$$

This formula is only approximate, since we have assumed the changes uniform; the actual relation between pressure and time is more complex. To this approximation U_1 is proportional to P, to $1/T$ and to $1/\lambda^2$.

Resal's formula [7] gives $\lambda = (Eh/2R)^{\frac{1}{2}}$, where E is the elasticity of the wall, of thickness h, and R is the inside radius. We then have from (2) that $U_1 = n\,(2PR/TEh)$ or, putting $2Rn/h = \alpha$, we have

$$U_1 = \alpha P/TE. \tag{3}$$

This relationship between the parameters is somewhat rough, since α varies a little (because of friction, etc.); it shows that U_1 is directly proportional to P, to $1/T$ and to $1/E$. The elasticity enters because any attempt to force more blood into the more distant parts of the artery encounters a resistance from the blood already there; blood is nearly incompressible, so fresh blood can enter the distant parts only if the arteries dilate. The volume of blood that can be accommodated increases with the arterial elasticity; so also therefore does the acceleration of the blood.

The rate at which the pulse wave propagates greatly exceeds the actual rate of blood flow, since it increases with the rigidity; the more rigid the artery the faster the pulse wave propagates, but the more slowly the blood moves, and conversely.

The arteries are not unlimitedly extensible; the greater the distension the less the extensibility, so the rate of flow falls, while the speed of the pulse wave rises. This is why blood flow rates are reduced in arterial sclerosis or in hypertension. In some

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states of hypertension or of reduced arterial tone, and if the systolic pressure rise increases, the acceleration of the blood is much increased; this causes sounds to be heard in the arteries even before the cuff is applied. The systolic flow rate is usually

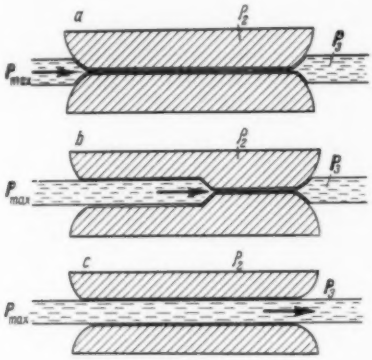

FIG. 3.

insufficient to cause arterial sounds; the cuff produces conditions favourable to increased flow rate. The ratio between wave and flow speeds is greatly altered in the section enclosed by the cuff. There is virtually no blood in the compressed section if the cuff pressure exceeds the diastolic pressure; if the pressure is less than the systolic the blood flows in spurts during the systolic phase. The pulse wave strikes the near end of the closed section and forces some blood into it (Fig. 3); the motion of the blood and the propagation of the pulse wave here run parallel and the speed is much below that of the pulse wave in an unoccluded artery (but much above that of the blood).

The systolic increase in flow rate depends on how much the artery stretches during systole; the increase in volume is normally small, but is much larger in an occluded artery, since the initial volume is almost zero. The volume and speed of the blood entering a section of an artery in systole are greatly increased if the section is occluded by a cuff. Cut arteries show spurts at systole because the liquid which would normally oppose the motion is absent. The blood also spurts through a section occluded by a cuff, but it has to enter a vessel already filled with blood on the far side. The speed and mass of the moving blood increase as the occluded section is opened up during a spurt; this large mass of fast-moving blood strikes the blood on the far side of the cuff and causes a shock which is heard as a sound. We now examine the relationships mathematically.

Let P_2 be the pressure of the cuff and P_3 the blood pressure at the far end of the cuff; P_{max} and P_{min} are as before. The pulse pressure wave reaches the closed section (Fig. 3*a*); some blood enters the closed area and overcomes the air pressure in the cuff* (Fig. 3*b*). The blood begins to enter the far section of the artery and

* The pressure to be overcome is in fact less than that of the air in the cuff by an amount dependent on the elasticities of the arteries and soft tissues; these effects are small, however, so the pressure is close to that of the air in the cuff.

has to overcome the pressure of the blood there (Fig. 3*c*). Let us consider the simplest case, where P_2 is close to P_{min}; let us suppose that T is unchanged; then p, and hence the acceleration a_2 of a mass m of blood in a length l is the same as before ($a_2 = a_1$). The speed U_2 of the blood in l is just a_2t_2; but t_1 is very short in an unoccluded artery, since $t_1 = l/\lambda$. The pressure is transmitted through the occluded section by the moving blood, however, so t_2 is inversely proportional to the rate at which the blood passes through the occluded section; hence $t_2 > t_1$. Taking the initial speed at the start of the length l as 0, the mean speed over the length U_m is just $U_2/2$. Then $t_2 = 1/U_m$, or $t_2 = 2l/U_2$. Now $U_1 = a_1 l/\lambda$ in the open artery; in the closed artery $U_2 = a_2t_2$, or $U_2 = a_2.2l/U_2$. Hence $a_1 = U_1\lambda/l$ and $a_2 = U_2^2/2l$; if $a_2 = a_1$ then $U_1\lambda/l = U_2^2/2l$, or $U_2 = (2U_1\lambda)^{\frac{1}{2}}$. This equation applies if $P_2 = P_{min}$; if $P_2 > P_{min}$ the resistance is larger and so the speed is reduced. If $P_2 > P_{max}$ the artery remains closed; the difference $P_{max} - P_2$ determines the flow rate in the occluded section, which will therefore be zero. Thus U_2 will change from $(2U_1\lambda)^{\frac{1}{2}}$ to 0 as P_2 increases from P_{min} to P_{max}.

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As friction has been neglected we can assume that the increase in speed is proportional to the pressure difference, so that

$$(P_{max} - P_2)/P = U_2/(2U_1\lambda)^{\frac{1}{2}} \text{ or } U_2 = (P_{max} - P_2)(2U_1\lambda)^{\frac{1}{2}}/P.$$

This gives the velocity attained at the end of the length l; if the cuff covers several sections of total length L ($= \Sigma l$) the resulting final velocity U_2' is given by ΣU_2, or

$$U_2' = (kL/P)(P_{max} - P_2)(2U_1\lambda)^{\frac{1}{2}}$$

where k is a coefficient of proportionality. However, the viscosity of a real liquid r cannot be neglected; the resistance encountered is proportional to r and L, and inversely proportional to the area of cross-section S. Hence the real speed U_2'' is somewhat different from U_2' and is given by

$$U_2'' = (\delta kL)(P_{max} - P_2)(2U_1\lambda)^{\frac{1}{2}}/P \tag{4}$$

where δ is a frictional coefficient dependent on r, S and L; $\delta = qS/rL$.

Equation (4) can be simplified somewhat by replacing U_1 by $\alpha P/TE$ from (3), and λ by $(Eh/2R)^{\frac{1}{2}}$ or $\chi E^{\frac{1}{2}}$, where $\chi = (h/2R)^{\frac{1}{2}}$, from Resal's formula:

$$\begin{aligned} U_2'' &= (\delta kL)(P_{max} - P_2)(2\alpha\chi P/TE^{\frac{1}{2}})^{\frac{1}{2}}/P \\ &= (\delta kL)(P_{max} - P_2)(2\alpha\chi/PTE^{\frac{1}{2}})^{\frac{1}{2}}. \end{aligned}$$

Putting $\delta k(2\alpha\chi)^{\frac{1}{2}} = \beta$ we have

$$U_2'' = \beta(P_{max} - P_2)L/(PT\sqrt{E})^{\frac{1}{2}}. \tag{5}$$

The blood already has a speed U_1 when it reaches the section under the cuff, so the final velocity is given by

$$U'' = \alpha\frac{P}{TE} + \beta\frac{(P_{max} - P_2)L}{(PTE^{\frac{1}{2}})^{\frac{1}{2}}}. \tag{6}$$

The pressure P_3 past the cuff is determined by the venous pressure in the extremity and is usually lower than the pressure in front of the cuff; hence the blood which penetrates under the cuff experiences a pressure difference $P_{max} - P_3$ during a time t_3. The resulting additional velocity is

$$U_3 = \gamma(P_{max} - P_3)t_3 \tag{7}$$

where γ depends on the friction, length, etc. The final velocity reached on the far side is thus

$$U = \alpha \frac{P}{TE} + \beta \frac{(P_{max} - P_2)L}{(PTE^{\frac{1}{2}})^{\frac{1}{2}}} + \gamma(P_{max} - P_3)t_3. \quad (8)$$

Equation (8) is not strictly exact, since α, β and γ are not constants, but it illustrates the general relationships between the quantities.

We have shown that the sounds heard in the "noise phase" are not pure noise; each sound in this phase begins with a brief tone which rapidly merges into a periodic noise. The tone and the noise fuse into one on auscultation and are heard as noise alone; the brief tone is entirely masked by the following noise. The noise is produced by the blood which gets under the cuff after the portion that is responsible for the shock which causes the tone; the duration of this noise depends on the amount and speed of the blood. The speed increases as P_2 decreases, and the time taken for the blood to pass under the cuff decreases. The initial tones are strengthened and the noise is weakened; Korotkovskii's third phase is reached.

If the artery is not completely closed even during diastole the blood flows continuously; the conditions for the tone to arise are lost. The loud tones of the third phase are replaced by the quiet ones of the fourth; the tones become quieter as the pressure is released. The equation governing the flow during the noise phase is

$$U = \alpha \frac{P}{TE} + \beta \frac{(P_{max} - P_2)L}{(PTE^{\frac{1}{2}})^{\frac{1}{2}}} + k \frac{(P_1' - P_3')S_1}{L}. \quad (9)$$

The first and second terms in (9) are the same as in (8), but the third is somewhat different; it represents the conditions responsible for the noise.* The equation for the fourth phase can be put as

$$U = \alpha PL/TE_1 \quad (10)$$

which resembles (3); the difference is that E_1 (for the partially occluded artery) is less than E (for a normal artery) and applies only to a short length L. Equations (9) and (10) are deduced as for (8); lack of space prevents the proofs being given. All are theoretical equations needing experimental verification; some special tests have been done with recording apparatus to check them. The theoretical arguments were found to be confirmed; the sounds are produced by the physical conditions which result from compressing the arteries, when the flow rates are greatly increased. The sounds also depend on the arterial elasticity, the venous pressure beyond the cuff, the time taken to eject the blood from the heart, etc.; they can therefore be used to study other aspects of haemodynamics as well.

SUMMARY

(1) Arterial sounds result from the increased kinetic energy of the blood at systole; the systolic acceleration and the extent of transformation of potential energy into kinetic are greatly increased by applying a cuff.

(2) The arterial sounds vary in loudness with the duration of systole, the difference between the pressure of the blood at systole and the cuff pressure, the pulse wave pressure, the arterial elasticity, and the venous pressure past the cuff.

*k is a coefficient of proportionality, P_1' is the pressure in the artery in front of the cuff at the start of the noise, P_3' is the pressure past the cuff at this time, and S and L are as before.

(3) The arterial sounds can be used to study haemodynamic phenomena other than arterial pressures, e.g. the duration of systole and arterial elasticity.

Translated by J. E. S. BRADLEY

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CAPACITANCE AND RESISTANCE OF LAYERS IN FROG RETINA*

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WE need to know the capacitance and resistance of the retinal layers in any study of the retinal potentials that are caused by stimuli; these primary data are almost entirely lacking from the literature. There is but one paper on the electrical properties of the eye, and that only partly fills the gap; Brindley [1] has passed square current pulses through frogs' eyes and has measured the potential drop across the eye as a whole, and across the residue when the retina has been removed, together with the pigmented and vascular layers. He calculated the resistivities of these structures from the results; he also detected structures that lie some 200 μ away from the vitreous humour and have about half the total resistance of the retina (250 Ω out of 500 Ω); he did this by inserting microelectrodes in the various retinal layers. He called the layer in which these structures lie the R-membrane; he concluded from histological data that this layer was the outermost membrane.

The retina distorted (rounded) the square pulses, as though there were capacitances in parallel with the resistance. No such effect was found when the microelectrode pierced the R-membrane. The mean time-constant of the combination was 10 msec, from which Brindley calculated the capacitance of the membrane as 100 μF/cm^2; this greatly exceeds all other values at present known for cell membranes (Eccles gives 2·5 μF/cm^2 for motor neuron membranes [2]. The mere size of the difference demands an experimental check; Brindley also gives only the resistances of the R-membrane and of the retina as a whole (with the former approximate), as

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he did not measure the resistances of other layers. Our paper deals with more detailed measurements of resistance and capacitance for various retinal layers.

In our first set of tests we deduced the resistances of the layers from the way the resistance of a microelectrode circuit varied as the electrode was pushed deeper into the retina. In our second set we used Brindley's method to measure the resistances of the layers in a radial direction. In our third set we dealt with how the d.c. potentials of the layers change, especially near the R-membrane. These latter data we combined with histological studies to establish some structural features of the membrane.

We used liquid microelectrodes 0·5–1·5 μ in diameter that were filled with 3 M KCl; these were joined to a cathode-follower system that we have described before [3]. The electrodes had resistances of 10 MΩ, which gave an input time-constant of 30 μsec; the grid current in the input valve was not greater than 2×10^{-12}A. The output from the cathode follower passed direct to an ENO-1 oscilloscope, which had an amplifier with constant gain from 0 to 10^5 c/s, and with an overall sensitivity of 3 mV/mm.

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1. THE RESISTANCE OF THE MICROELECTRODE IN VARIOUS RETINAL LAYERS

The principle used in this set of tests was as follows. There are two main components to the resistance of a microelectrode that has been placed in a salt solution, namely the resistance of the electrode proper, which is that of the liquid in the capillary, and that of the liquid near the tip of the electrode. The first component cannot change much when the electrode is put into a medium denser than the salt solution (i.e. that within the retina); all we can expect is that the ions will diffuse slowly out of the capillary, and that this will dilute the solution at the tip less than it will when the electrode is put into Ringer. Hence we must assign to the second component any increase in resistance that we find on inserting the electrode into the retina. Resistance measurements thus give us data on the conductivities of the tissues.

We used isolated eyes from *Rana temporaria* and *Rana ridibunda* and placed them in a moist chamber when we had removed the cornea, the outer sheath, the crystalline lens and part of the vitreous body. We brought the tip of a non-polarizing (Ag–AgCl) electrode into contact with the sclera or vitreous body, and inserted the microelectrode (fitted in a micromanipulator) into the retina from the vitreous body side. It was usually easy to see when the tip reached the retina if a binocular magnifier was used. The blood vessels in the retina could be seen if the lighting was good, and so could the nerve fibre bundles that converge to the blind spot. The microelectrode flexed the surface when it touched it, so the moment of contact was easily noted. In many cases we detected the moment of contact from the negative-going step seen on the screen of the oscilloscope, which appeared to be caused by the mechanical pressure. The moments of contact found in these two ways were always very close together, although the second is probably the more exact.

We measured the resistances in the usual way [3]. The calibrator passed a square current pulse of fixed size direct to the input of the cathode follower; the same pulse was then fed to the input via the microelectrode. The deflections seen on the oscilloscope were compared; the resistance of the microelectrode was easily calculated if the input resistance was known. Table 1 gives the results from two experiments;

the retina gave resistances much larger than did the vitreous body or the Ringer. The changes at depths up to 60–70 μ were not large, but the subsequent rise was; the maximum resistance was found at 175–200 μ. It was found that the resistance suddenly dropped almost to the initial value when the microelectrode was pushed only a few microns beyond this point of maximum resistance; there was usually also a simultaneous negative-going step in the potential, which was sometimes as much as 50–60 mV. The step was usually very short-lived, and the potential returned nearly to the initial level within 1–3 sec (these changes in potential are dealt with below). The resistance did not usually change very much when the electrode was pushed in a further 40–50 μ.

TABLE 1. RESISTANCE VALUES FOR RETINAL LAYERS

Depth penetrated from the vitreous-body side (μ)		0	25	50	75	100	125	150	175	200	205	210	225	250
Resistance values (MΩ)	I	5·2	5·7	6·8	9·5	8	9·1	11·3	16·3	24·5	—	5·2	—	—
	II	20	20	20	41	62	70	80	100	110	20	—	20	20

The retina showed a resistance higher than that found for the vitreous body, but the value varied from point to point; different retinas also showed different values. The resistance of the retina might be only twice that of the vitreous body, but was often four or five times as great, as Table 1 shows.* The resistance returned stepwise to the initial value at a depth that varied from 150 to 225 μ (usually 175–200 μ).

The rise in resistance must have been caused entirely by the increased resistivity of the medium in the retina. Now the resistance of the liquid round the tip is only part of the total resistance (the value for a 0·5 μ tip in Ringer is calculated to be about 1 MΩ), so the incremental resistance found in the retina is much more than five times the resistance found in Ringer. The first example in Table 1 (1 μ tip) gives an increase of fifty-fold, while the second (0·5 μ tip) gives one of about a hundred-fold. The values are only rough, since we did not measure the internal diameters of the tips exactly, but they reflect the order of the effect correctly.

One objection to such calculations is that we neglect the possibility of the lumen's being partly blocked by tissue. It would seem that such blocking does not occur, since at a certain depth (here 200 μ) the resistance, which had previously increased, dropped suddenly back almost to the initial value. The resistance also stayed fixed at the value for Ringer when the electrode was then withdrawn, since the tip was then in a channel that was full of a liquid of composition close to that of Ringer. The increased resistance would have persisted had the electrode been blocked. In some cases the tip was in fact blocked; this happened, for example, if the electrode was pushed in 300–350 μ, to contact the sclera. The resistance then sometimes rose by ten-fold or more (if the tip did not break) and stayed high when the electrode was withdrawn.

* Brindley [1] has in some cases measured the resistance with the microelectrode at various depths, but did not find changes of more than 2 MΩ (i.e. 30–40 per cent).

The increased resistance we have found within the retina may be caused by the numerous cell membranes there. The nerve cells lie very close together in the layer that contains the bipolars and receptor nuclei, so the resistance round the tip may be large if the tip lies between two cells or rests on a membrane. It may be that the resistivity of the medium between the retinal cells is higher than that of the vitreous body or than that of Ringer. We found that the resistance showed peaks if the electrode was pushed very smoothly into the retina, but the value was at all times larger than was found with Ringer.

There is some sort of boundary (which we shall show is the outer membrane) at 175–200 μ from the inner surface of the retina; this boundary divides the high-resistivity medium within from the low-resistivity one without (which contains the rods and cones). We know that pigment enters the gaps between the rods (the retinomotor effect) when the eye is adapted to very strong light, so the gaps between the receptors must be filled with fluid. This intercellular fluid has a composition close to that of Ringer.

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2. RADIAL RESISTANCE AND CAPACITANCE IN THE RETINA

We used Brindley's method, somewhat modified, in the next set of tests. Fig. 1 shows the system used.

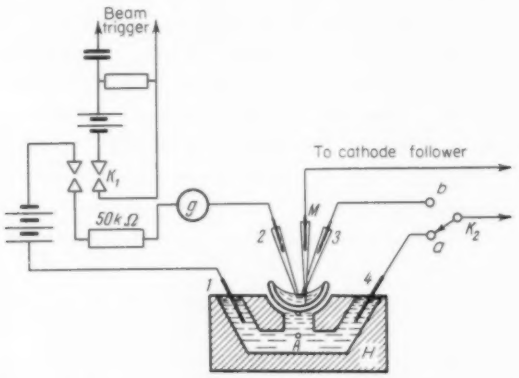

FIG. 1. The circuits used in measuring radial resistance and capacitance on frogs' retinas. Explanation in text.

The isolated eye was placed in a recess in the special wax holder *H*. The channels bored in the wax held Ringer that joined the sclera to the two Ag–AgCl electrodes *1* and *4*; on the vitreous body side there were two further electrodes, also non-polarizing, which were made from wide glass capillaries filled with agar that had been made up with 5 per cent NaCl solution; silver wires completed the electrodes. The microelectrode *M* was pushed into the retina with a micromanipulator. Electrodes *1* and *2* passed square current pulses radially through the retina when the left contacts of switch K_1 were closed; the galvanometer *g* measured the current that flowed. The right contacts triggered the time-base in the oscilloscope; they closed somewhat

earlier than did the left ones, so that the current pulse should begin at a time after the sweep started; this made photographic recording convenient.

The potential difference that appeared across the retina was measured with the microelectrode working in conjunction with one of the pair of electrodes *3* and *4*. The resistances of the layers were calculated from the potential differences and the current (which varied from 50 to 150 μA); the capacitances were calculated from the shape that the pulse took. Figure 2 illustrates a typical experiment. The left side shows records obtained with a 50 μA current pulse, for recordings taken from the point *a*, i.e. between the microelectrode and electrode *4*, which was joined to the sclera (Fig. 1). The retina, the vascular and pigmented layers, the sclera, and part of the channel in the vessel that held the Ringer, were between the lower electrode and the microelectrode when the latter was at the surface of the retina. The potential difference found at *a* was thus caused by all the layers. It is clear that the rise time is finite, as if a capacitance had been connected across some part of the resistance.

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The potential dropped slightly without the curve's changing shape as the microelectrode was inserted to a depth of 190 μ, but any further movement caused a sharp change in the curve. The voltage was almost halved, and the rise time became very short.* No further change occurred when the microelectrode was inserted a further 60 μ. The curve was of the original height and shape when the microelectrode was returned to the surface.

The right side shows the potentials that were recorded from the same current pulses, when we used the microelectrode and the top electrode *3*, which was inserted in the vitreous body (position *b*). There was naturally no potential difference when the microelectrode was at the surface, since both electrodes were in the vitreous body; there was also almost no difference down to depths of 120 μ. There was, however, an appreciable rise at 125 μ, which often occurred stepwise.† The rise was still greater at 175 μ; there was a sharp change at 190 μ, as with position *a*. The pulse became rounded and of much greater height.

The potential drop across the whole preparation did not change during the tests, so the sum of the two potential differences should have been constant, as is actually the case, as columns *a* and *b* of Fig. 2 show (within the errors of measurement). Table 2 gives the resistances measured from the internal surface of the retina to various depths; it has been compiled from the results of Fig. 2. It is seen that there is a sudden rise of 500 Ω at 190 μ. The total resistance of the entire eyeball is found by subtracting from the total resistance the resistance of the Ringer in the vertical channel (the former is found from the potential difference measured from Fig. 2*a*1). The resistance of the Ringer is about 185 Ω (by calculation, and from the height of the step in Fig. 2*a*7); the total resistance is 835 Ω. This gives the resistance of the eyeball as 650 Ω, so the vascular and pigmented layers, with the sclera, account for about 80 Ω.

There is thus a layer some 190 μ away from the inner surface which, although it is very thin, accounts for some 80 per cent of the total radial resistance, and has a certain capacity. Brindley has called this structure the R-membrane and has concluded that it is the outermost membrane.

*A small peak above the final level is seen on the trace; this is caused by the parasitic capacity between the exciting and recording circuits.

† The polarity of the pulse is of course the reverse of that for position *a*.

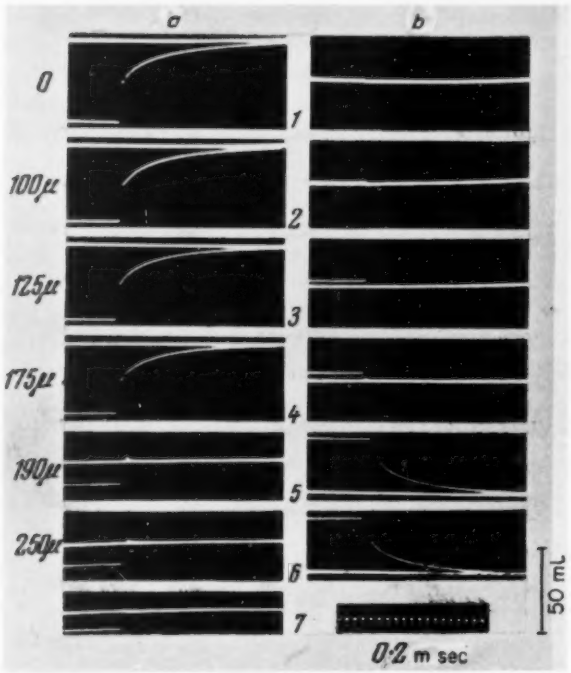

FIG. 2. Voltage records from various layers in the retina produced by square current pulses. On the left: recorded from the microelectrode and electrode *4* (Fig. 1*a*). On the right: recorded from the microelectrode and electrode *3*. (*b*): The figures on the left are the depths in microns. The *a7* curve is that recorded with a similar pulse after the preparation had been removed and the microelectrode had been put directly in the Ringer.

The typical thicknesses of the retinal layers were found to confirm this conclusion. In some cases we recovered the retina after the measurements and carefully sectioned it with a razor under a dissecting microscope, using roughly the area that had been examined with the microelectrode. The sections that resulted were thick (100–200 μ), but the edges of the layers were well seen. The thicknesses of the layers could easily be measured when they had been stained with methylene blue. The distance from the internal surface of the retina to the outermost membrane was almost exactly

TABLE 2. RESISTANCE VALUES FOR RETINAL LAYERS

Depth of microelectrode (μ)	0	50	75	100	125	150	175	190	225	150
Resistance from surface of retina (Ω)	0	0	18	18	46	74	74	570	570	570

Note: *R* for the whole eyeball is 650 Ω, while *R* for the preparation and the vertical channel is 835 Ω.

equal to the distance at which the sudden change in resistance was found. The value was on average about 15–20 μ less than that given by the microelectrode results; the effect is caused by the microelectrode's bending the retina, particularly the outermost membrane. The layers became somewhat thinner near the edge of the retina, which corresponds to the reduced depths that are found at the edges with the microelectrode.

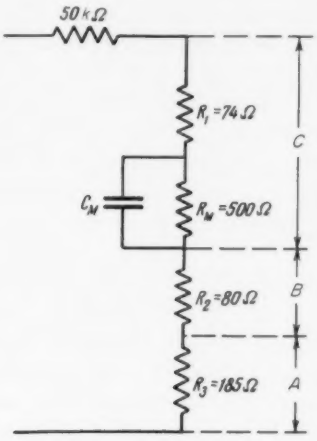

FIG. 3. The equivalent circuit that describes the electrical properties of the preparation. The 50 kΩ resistance in the input circuit (see also Fig. 1) is designed to keep the current constant. The rest of the circuit is explained in the text. A = Ringer in chamber, B = sclera, and vascular and pigmented layers, C = retina.

We have drawn up the equivalent circuit of Fig. 3 from these data. Here R_1 is the resistance between the inner surface of the retina and the membrane, R_2 is the resistance of the pigmented and vascular layers and of the sclera, R_3 is the resistance of the Ringer in the vertical channel, and R_m and C_m are the resistance and capacitance of the membrane. We have not included the resistance of the layer of internal and external parts of the receptors (which layer lies between the pigmented layer and the membrane) because it is so small as to be almost undetectable by our method.

This circuit explains very well the curve shapes that we found. The first almost instantaneous rise in potential difference (Fig. 2*a1*) occurs across R_1, R_2 and R_3. The rise time is determined by that of the cathode follower only (here 30 μsec). The subsequent more gradual rise occurs because a potential difference gradually builds up across the membrane. The capacity was calculated from the time-constant; in our case (Fig. 2) it was 0·75–0·80 msec, so the capacity was

$$C_m = \tau/R_m = 1{\cdot}5\text{–}1{\cdot}6\ \mu\text{F}.$$

The results given in Fig. 2 are more or less typical, though the resistance and capacitance values may vary widely from one preparation to another. The resistance of the retina varied from 400 to 1000 Ω, with the membrane responsible for from 60 to 90 per cent of this. The capacitance varied from 0·8 to 2·0 μF (it usually lies between 1·3 and 1·6 μF). The differences are accounted for partly by differences in

the sizes of the eyes, but not entirely. Repeated measurements on any one preparation gave closely similar results, but the resistance and capacitance both dropped greatly if the eye was allowed to stand for a long time, or was in a poor state. A few drops of 90 per cent alcohol applied to the eye caused the time-constant to decrease within 15–20 min, and to vanish almost completely after 40–50 min. Light, even if of high intensity, had no appreciable effect on the electrical properties.

Taking the area of the retina as 0·5 cm^2 (Brindley gives 0·38 cm^2), we find that the capacitance per unit area was about 3 $\mu F/cm^2$, and the resistivity about 150–350 Ω/cm^2. The latter value is somewhat larger than Brindley's (100 Ω/cm^2), while the capacitance is about a thirtieth of Brindley's. It is difficult to explain this large difference; it cannot have been caused by our using a current larger than Brindley's (50 μA instead of 16·7 μA). Our data, and Brindley's, show that such currents have almost no effect on the electroretinogram, and do no real damage to the outermost membrane. It is a pity that the pulse recordings that Brindley gives were taken with a time-base so slow that it is impossible to estimate their time-constants.

3. THE CHANGES IN THE D.C. POTENTIAL ON INSERTING A MICROELECTRODE IN THE RETINA

The potential went negative by several millivolts when the microelectrode touched the retina. The usual process of inserting the electrode in steps also led to the electrode becoming negative relative to the vitreous body or to the sclera, but the potential difference returned in part to the initial value when the electrode was at rest. The potential changes increased in size with the size and speed of the movements. Thick electrodes showed a larger effect than did thin ones; the effects were especially large if the tip of the electrode was broken. The cause was probably the mechanical pressure of the electrode on the tissue. It was thus very difficult to establish the true potential differences between layers in this way.

Other changes in the potentials were also found which were undoubtedly physiological in origin. A negative-going step of height 30–60 mV was often found even when the microelectrode was inserted very slowly and smoothly. The difference usually soon vanished, but sometimes stayed constant for a few seconds. In the latter cases the potential returned to the initial level if the tip was pushed in a further 5–15 μ, or was withdrawn. This step effect was found at 15–30 μ and at depths varying from 80–100 μ to 150–200 μ (just in front of the membrane), which levels correspond to the layer of ganglionic cells, and to the layers of bipolars and of rod nuclei, respectively. The layer between 30 and 80 μ (the internal synaptic layer) usually showed no potential steps. This demonstrates that these negative steps were caused by the electrode entering cellular bodies. We must point out though, that light stimuli did not change the intracellular potential at all (the electroretinogram and the extracellular potentials showed the usual effects, of course).

The R-membrane also gave these negative steps when it was touched; the values varied, but were often 50–60 mV. The fall could be rapid or slow. In some cases the potential soon returned to the initial level; in others a residual potential of 5–15 mV persisted for up to a minute. The potential returned rapidly to the value found before the membrane had been reached when the electrode was pushed in any further, and changed no more right up to the back of the retina (i.e. for another

60–70 μ). We usually found no changes as the electrode was withdrawn from a final depth of 250–275 μ. We may thus conclude that there is no major potential difference across the membrane.*

If the electrode was withdrawn by 5–15 μ as soon as the potential change caused by contact with the membrane was detected, it was found that the potential (40–50 mV negative) persisted for seconds or even for minutes. This negative potential could be reproduced several times by moving the electrode backwards and forwards, though the value was much reduced at each repetition, presumably because of the damage caused. It appears that there is a zone of negative potential at the level of the outermost membrane; the membrane itself seemed too thick to be able to contain such a layer within itself.

The shape of the voltage pulse recorded with the tip in this zone gave us additional data on this negative zone. The time-constant was very much larger than was found when the membrane had been pierced (Fig. 4). The resistance (wave height) was intermediate between the values found in front of and behind the membrane (Fig. 4*a*, *b* and *c*), so it seems that the negative zone lies inside the membrane. The magnitude of the negative potential and the time-constant usually increased together; the two also decreased together as time passed. The shape of the wave was independent of the sense of the current.

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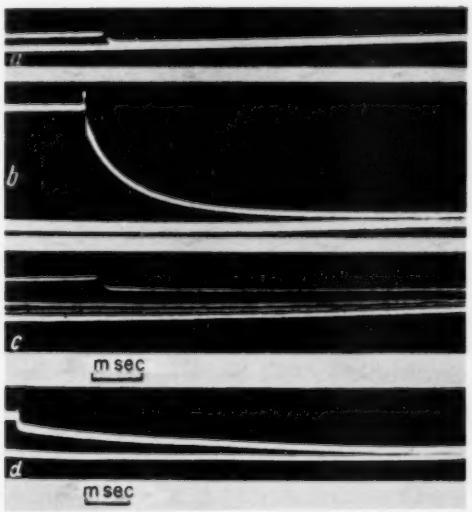

FIG. 4. The potential differences recorded (*a*) before the microelectrode has reached the outermost layer, (*b*) after it has pierced it, and (*c*) when the microelectrode is in the negative potential layer at the membrane level; (*d*) is the same as (*c*), except that the sweep rate is about one-third of the previous value. The microelectrode and electrode *3* were used (see Fig. 1).

* Brindley [1] considers that the potential difference across the R-membrane is the main component of the resting potential in the eye, though Noell's data [4] indicate that the pigmented epithelium is mainly responsible for the resting potential, in the rabbit at least.

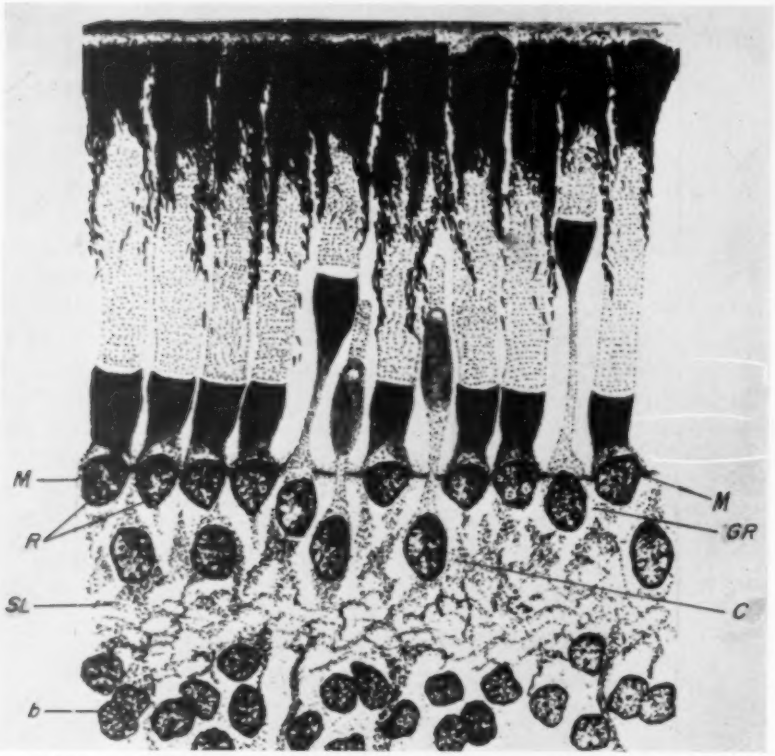

FIG. 5. The retinal structure in *Rana ridibunda* in areas near the outermost membrane. *R*—rod nuclei; *GR*—"green rod" nuclei; *C*—cone nuclei; *M*—outer membrane; *SL*—outer synaptic layer; *b*—bipolar nuclei. Fixation: ether–formalin. Stain: haematoxalin–eosin.

The time-constant found in the negative zone was larger than was found at any other point; this shows that the effective circuit of large *RC* was not connected in series with the membrane. This conclusion agrees with the fact that no potential step was found in some cases when the membrane was reached; the time-constant was also short in such cases. We may suppose that this structure with a negative potential is not continuous throughout the membrane, but exists as separate areas which in sum cover more than half of the surface (since the potential steps were found in a majority of the tests).

We can establish with some certainty what this structure is from histological data. Fig. 5 shows a radial section of a frog's retina that includes this membrane.* The nuclei of the cones and of the green rods are seen between the membrane and the outer synaptic layer. The nuclei of the ordinary rods lie at the level of the membrane. Electron micrographs [5] of guinea-pig retina have shown that the membrane is made up of ring outgrowths from the cellular sheaths at the bases of the internal parts of the rods. These parts are completely taken up by the nuclei in the frog and are, as it were, included in the membrane; they take up much of its area.

* I am indebted to I. A. Utina of this laboratory for this photograph.

It is still not clear why a constant potential difference was found on piercing the rods' nuclei (if the membrane is actually these nuclei), and why none was found in response to light stimuli. The same effect is found on piercing cell bodies in the bipolar layer; again, there is a 60 mV negative potential that is unaffected by light. Brindley has found the same [1], and similar effects have been found with fish retinas [6]. The effect may be caused by some structural peculiarity in the retinal nerve cells. The nuclei take up almost all of the space in the cells in the bipolar and ganglionic layers, and leave only thin rims of cytoplasm. It is thus very difficult to insert the microelectrode in the cytoplasm, as is done, for instance, with motor neurons; the electrode enters the nucleus the moment it has pierced the cell membrane. Further careful studies are needed.

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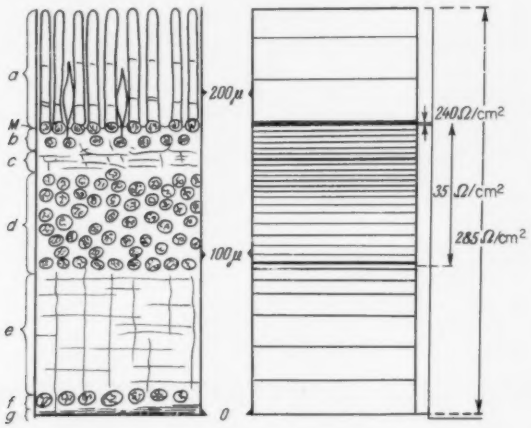

FIG. 6. Diagram of the radial distribution of resistance and of the various layers. *a*—Layer of external and internal rods and cones; *M*—external boundary layer; *b*—external nuclear layer (primitive rods and cones); *c*—external synaptic layer; *d*—internal nuclear layer; *e*—internal synaptic layer; *f*—layer of ganglionic cells; *g*—layer of nerve fibres. Further explanation in text.

CONCLUSIONS

Figure 6 may be taken to summarize the results from the first and second experimental sections. We must first point out that the scheme illustrates only the general form of the results, and does not reflect the rather large differences between different experiments. The left column gives the approximate positions of the various retinal layers in the frog; it has been compiled from sections of our material that have been stained with methylene blue. The right column gives the radial resistances of the layers; the spacing of the lines approximately represents the specific resistance. The layer of bipolars and rod nuclei has a specific resistance that is some fifty to seventy times greater than that of Ringer. The numerical values relate to the tests of Fig. 2. We have already pointed out that these results are typical and are close to the mean values for the whole series.

The outermost membrane (that at 180 μ) is shown as a thick line; it has a resistivity of about 240 Ω/cm², which is 87 per cent of that for the whole retina; its capacitance

is about 3 μF/cm². The entire region between the membrane and the layer of nerve fibres has a resistivity of about 35 Ω/cm², of which almost all is assigned to the layer of bipolar nuclei, to the outer synaptic layer, and to the nuclear layer. The thicker lines shown opposite the synaptic layer and at the bottom of the bipolar layer indicate that there may be some structures at these points where the resistance rises sharply. The top structure (that at a depth of about 160 μ) has a certain capacity, since in some cases the wave shape showed a certain rounding at this level. This structure may be the outer synaptic layer, or some part of it. Ringer has a resistance (in the same thickness) that is only a half or a third of that of the layer made up of the receptors' outer and inner segments, which layer has a resistivity of 1·5–2 Ω/cm².

Translated by J. E. S. BRADLEY

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* There is a translation into English of this work.

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THE EFFECTS OF TEMPERATURE ON THE ACTION POTENTIALS OF FROGS' NERVES*

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THE effects of temperature on action potentials have been studied since the cathode-ray oscilloscope was first used in physiology [1–10]. In every case it was found that cooled nerves showed action potentials of much longer duration, but opinions on the effects of temperature on the amplitudes have so far been most conflicting. Most workers who have used frog nerve trunks [1–3, 5, 9] have found that the potentials at first fall slightly, but then more rapidly, as the nerves are cooled. Gasser's data [1] show that the temperature coefficient of the amplitude changes from 1·5–2 at 15–25° to 10 or more at about 5°. Tasaki and Fujita [7] have found a similar fall with single nerve fibres from the toad. Contrary results have been found with frog [4] and octopus [8] nerve fibres; cooling caused a slight rise in amplitude. Mammalian nerves show a fall when group A fibres are cooled, but a rise when group C are cooled [6]. It has also been found [10] that frogs' nerves give potentials that do not vary between 1 and 31°.

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We have already shown [11] that the resting potentials of frogs' nerves change in two stages as the nerves are cooled. The resting potentials increase if the nerve is cooled by not more than 10–11°, but decrease if one cools the nerve further. We report here our work on the action potentials of frogs' nerves; the tests were done in the same way as for the resting potentials.

METHODS

We used sciatic nerves from *Rana temporaria*; the nerve was cooled generally, as in [11]. We arranged the isolated nerve on two pairs of silver electrodes that lay at the bottom of a deep moist chamber. The stimulator electrodes were 2–3 mm apart, the pick-up ones 16–18 mm apart; the two sets were spaced at from 5 to 15 mm. We ensured that we got single-phase potentials by destroying the distal end of the nerve more than 5–6 mm away from the proximal pick-up electrode. The nerve was greased with semi-liquid petroleum jelly to prevent its drying or being wetted by condensed water (water is bound to condense as the chamber is cooled).

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The signals were fed to a four-stage RC-coupled amplifier of input resistance 500 kΩ; an oscilloscope recorded the output from the amplifier. The stimulator was a single-pulse generator that gave pulses lasting 0·05 msec; its output was fed to a high-resistance low-ratio transformer that had its secondary joined to the stimulator electrodes. The stimulus artifacts were then slight, and no earthed plate was needed between the stimulator and pick-up electrodes; this plate can distort the action potentials seriously [12]. The stimuli were always above the level that gave maximal response.

The prepared nerve was put in Ringer for 30–60 min, and was then put on the electrodes in the chamber. The action potentials were recorded several times at room temperature; the chamber was then put in a bath of cold water and the potentials were recorded as the nerve cooled. The nerve was reheated by putting the chamber in a vessel that held water at room temperature.

RESULTS

In the first set of tests we cooled the nerve by 13–18° (from 20–23° to 5–7°). In nearly all cases we found that there was almost no change in spike height until the nerve had cooled by about 6–8°; then the height began to fall steadily. The fall from the initial value was 1·8 per cent per degree within the limits stated above. In some 20 per cent of cases we found that there was at first a small rise (never more than 2·5 per cent of the starting value), which never lasted for more than the first three measurements, during which the nerve cooled by only 2–4°. The changes were always fully reversible.

Figures 1 and 2 illustrate the results. Figure 1 shows action potential oscillograms for the nerve uncooled, for during the cooling, and for after the nerve had been reheated. Figure 2 shows the same data in graphical form. These results were found in forty-two tests in this set; in five tests only did we find different types of effect. In three cases we found no change on cooling the nerve, and in two a prolonged rise of 10 per cent over the first value.

We cooled the nerves to from +0·5° to −0·5° in another set of tests; these showed that frogs' nerves are most resistant to cooling, and that the nerves did not

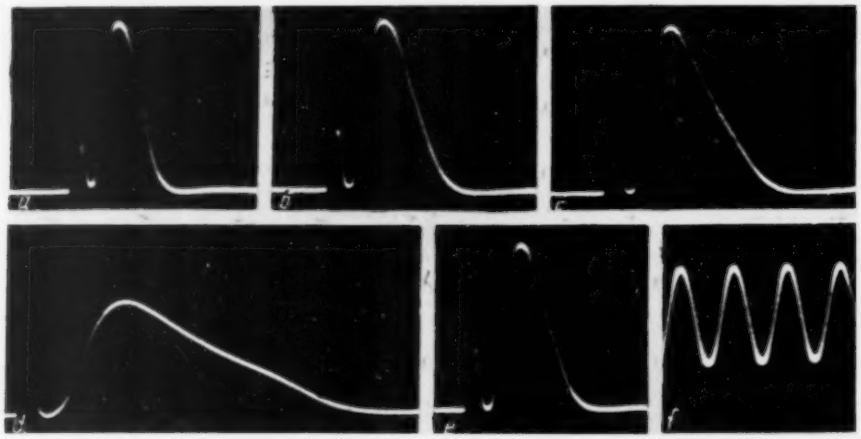

FIG. 1. The action potentials of a nerve trunk at various temperatures (experiment of 9 December 1957). (*a*) 16 hr 1 min, 19°; (*b*) 16 hr 7 min, 13·8°; (*c*) 16 hr 13 min, 9·6°; (*d*) 16 hr 41 min, 4·7°; (*e*) 17 hr 18 min, 16°; (*f*) 1000 c/s time marker.

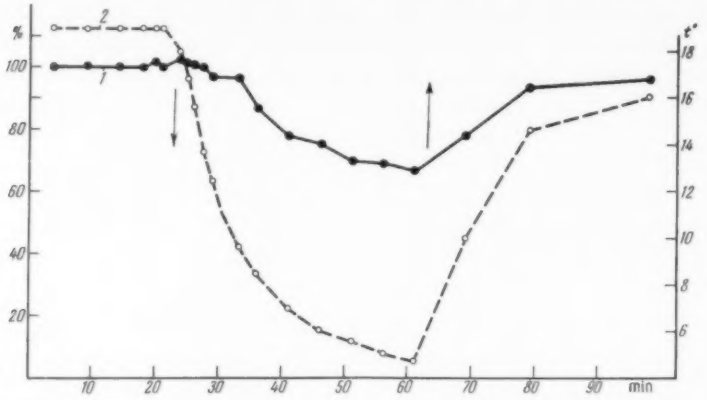

FIG. 2. The effects of temperature change on the peak action potential given by a nerve trunk (experiment of 9 December 1957). (1) Peak height as per cent of initial value; (2) temperature, °C. The arrows denote the start and finish of the cooling.

cease to be excitable even at these degrees of cooling. It has sometimes been found that the spike heights fall the more rapidly the greater the cooling; we found this also. The mean fall per degree was 3·5 per cent in the range from 20 to 0°, i.e. was larger than in the range from 20 to 5°C, but we never found a fall so great as Gasser found [1]. Gasser found temperature coefficients of ten and over at low temperatures, but we never found values larger than 2–2·5. The changes were nearly reversible, even at these low temperatures; Fig. 3 illustrates this claim.

The most notable aspect of the spikes that occur at low temperatures is that they lasted longer, as the figures show. The spikes began to last longer as soon as the cooling started, before the amplitudes showed any change. In some cases the rising and falling branches of the spikes changed in the same way when the cooling started;

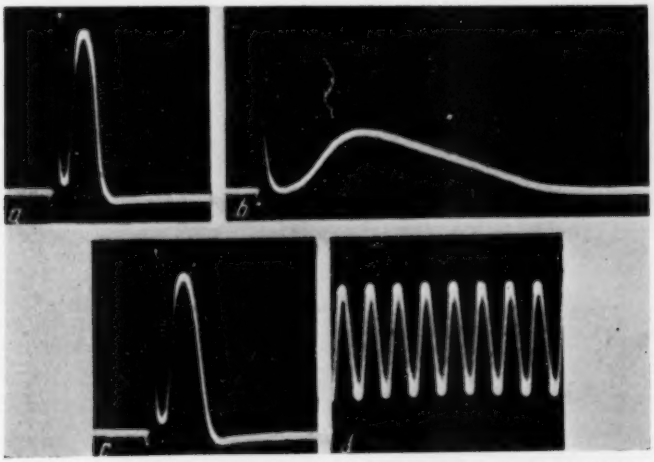

FIG. 3. As Fig. 1 (experiment of 8 October 1957). (*a*) 15 hr 24 min, 18·5°; (*b*) 16 hr 55 min, 0·5°; (*c*) 17 hr 50 min, 15·7°; (*d*) 1000 c/s time marker.

later the descending branches began to be steeper than the rising ones. In other cases the descending branches were steeper than the rising ones from the start. The rising branches increased in mean length by 20·7 per cent per degree in the range from 20–23° to 5–7°, the falling by 25·7 per cent. The times were 0·3–0·5 msec at 19–23° and 0·9–1·4 msec at 5–7° for the rising branch; the corresponding figures for the falling branch were 0·75–1 and 2·3–3·8 msec. Figure 4 shows how the spike time varies with the temperature; the results used are those of Figs. 1 and 2. The curve followed by the chamber temperature is also shown, as in Fig. 2.

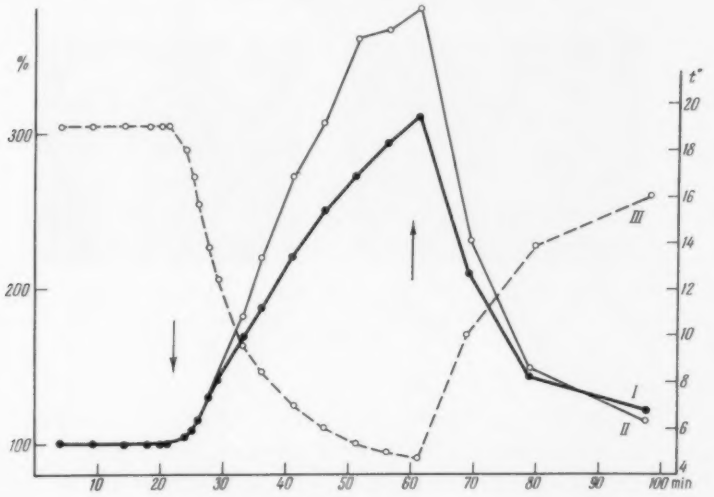

FIG. 4. The effects of temperature change on the rising (*I*) and falling (*II*) branches of a nerve-trunk action potential (experiment of 9 December 1957). The arrows denote the start and finish of the cooling. *III* = temperature, °C.

These data show that the action potentials varied with temperature in a way quite different from that we have found with the resting potentials. There is, in particular, no two-stage change in the spike duration, while the amplitude in general tends in the same direction. Even when there was initially a rise in spike height, the magnitude and duration of the effects were negligible relative to those found with the resting potential.

It may be objected that this lack of two phases to the changes in the action potential is but apparent, and arises only from effects on the common nerve trunk. This

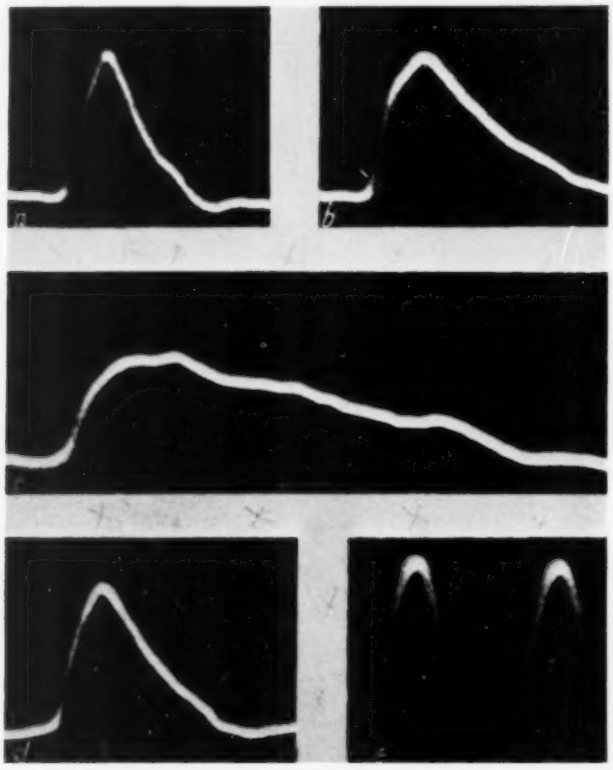

FIG. 5. The action potentials of a single nerve fibre at various temperatures (experiment of 17 October 1957). (*a*) 15 hr 0 min, 21·7°; (*b*) 15 hr 7 min, 13·5°; (*c*) 15 hr 30 min, 5·9°; (*d*) 17 hr 0 min, 18·6°; (*e*) 1000 c/s time marker.

objection is justified, because all who have studied the effects of temperature in nerve physiology have found that a cooled nerve conducts more slowly. Our data also show that this is so; the time interval between the stimulus artifact and the rising front of the action potential increased as the nerve was cooled. It is quite likely that nerve fibres differ in how their conduction rates vary with temperature; this would mean that the spread in the times taken by the pulses to travel along the various

fibres would increase as the nerve is cooled. This rise in the time spread would itself reduce the spike height and increase the duration.

We used single frog-nerve fibres to solve this problem, with phalangeal preparations made by Latmanizova's method [13, 14]. The preparation consisted of the 10th anterior root, the nervus ischiadicus and the nervus peroneus, dissected out as far as the terminal branch that innervates the short extensor muscle of the middle digit. The other fibres that innervate this muscle come from the 11th root. The action potential of a single fibre could thus be recorded when the 10th root was stimulated. The pick-up electrodes were 2·5–3 mm apart; the distal parts of the nerve were carefully destroyed, in order to get single-phase records. The method had the disadvantage that the action potential of the active fibre was shunted by the rest of the fibres in the terminal branch; the amplifier had therefore to have quite a high gain, and the signal was distorted somewhat by noise, as the records show.

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We see that a single fibre gave an action potential that varied with temperature in the same way as did the action potential from the common nerve trunk. The potential fell at first very slightly, and then more rapidly, as the nerve was cooled from 20°C to 5°; the mean fall per degree was 1·9 per cent. Single fibres sometimes gave a small rise at first, as did common nerve trunks, but the later fall very soon set in. The fibres gave spikes that began to last much longer as soon as the cooling started; here the falling branches showed more change relative to the rising ones than we have found with common nerve trunks; the mean increases in duration per degree fall were 31·6 per cent and 21·6 per cent respectively. The changes were all reversible. Figure 5 shows typical results for this set.

These tests on single fibres confirm our deduction [11] that the changes in the action potentials of the separate fibres are the main cause of the fall in the potential of the common nerve trunk. The fibres always stayed excitable when they were cooled to 5°; in one case only did the action potential vanish at 3·5° and just before this point the spike height had fallen by about 30 per cent of the initial value.

DISCUSSION

The resting potentials [11] and action potentials of nerves vary differently with temperature; the resting potential changes in two stages, whereas the action one does not. The action potential falls much more than does the resting one when the nerve is cooled; e.g. the action potential falls by 12–30 per cent from the initial value at 5–7°, and by 65–85 per cent at 0°, whereas the resting potential never falls by more than about 10 per cent. Many published data show the same; Bremer and Titeca [2, 3] found that the action potential fell very much more than did the resting one when a nerve was cooled. Lundberg [6] has found that group A fibres from mammals, when cooled, show a small fall in resting potential and a very much larger one in action potential, though group C fibres show rises instead of falls. Hodgkin and Katz [8] found a very small rise in membrane potential and a larger rise in action potential with squid giant axons. Fatt and Katz [15] found that various muscle fibres showed membrane and action potential changes that differed in direction: Nastuk and Hodgkin found the same [16] for frog muscle fibres. The membrane potentials fell slightly, while the action potentials rose, in these cases.

Gasser [1] compared his results with Verzar's [17] for the resting potential and

claimed that the facts contradicted Bernstein's ideas, which had till then held sway (i.e. that the action potential was just a simple membrane depolarization phenomenon). This conclusion has now been confirmed; Lorente de No [5] observes that the action potential must be dealt with as due to a new e.m.f. of sense opposed to that of the resting potential, and not as due to the resting potential's vanishing temporarily. Hodgkin's ideas [18] imply that the cell membrane becomes selectively more permeable, first to sodium ions and then to potassium ions, during excitation. The sodium ions enter the cell (rising phase), which then loses an equivalent amount of potassium ions (falling phase). Weidmann [19] concludes that the factors that determine the size and shape of the action potential are (1) the permeabilities to ions of the membrane, (2) the rate at which the permeabilities change in response to membrane potential changes, (3) the rates of active transfer of the ions, and (4) the distribution of the ions between the cell and the surrounding fluid. This last determines the membrane potential, in a final reckoning, but is only one of the factors that determine the action potential. Temperature affects membrane permeabilities and ion transfer rates particularly strongly [20]. This may explain why the action potentials change greatly in height and (particularly) in duration while the resting potential changes independently.

SUMMARY

(1) An isolated frog nerve gave an action potential that at first stayed the same as the nerve was cooled gradually, and that then fell steadily. In a few cases the peak potential rose briefly at the very start of the cooling.

(2) The action potential began to last longer right from the start of the cooling; the falling branch lengthened more than did the rising one.

(3) A single fibre showed the same size and type of changes in action potential in response to cooling as did a common nerve trunk.

(4) The action potential of a cooled nerve changed in a fashion distinct from that found for the resting potential.

(5) Published data are quoted to show why the action and resting potentials do not change in the same way.

Translated by J. E. S. BRADLEY

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AN ELECTROPHYSIOLOGICAL ANALYSIS OF HOW NON-IMPULSIVE EFFECTS SPREAD ALONG NEUROMUSCULAR STRUCTURES FROM X-IRRADIATED AREAS*

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IT is of interest to study the effects that originate in irradiated areas of the nervous system and appear in innervated tissues or in parts of the nervous system outside the irradiated area, especially in relation to the effects of ionizing radiations on the nervous system. We have reviewed the published data before [1]; there is evidence that some effects propagate along the nerve structures themselves [2–4]. We have then to find how effects propagate along nerve structures from irradiated areas.

The idea that pathological signals are emitted is based only in part on experiments on receptor-field irradiation [5, 6]; there are no data that show that signals are caused by the direct action of irradiation on nerve centres and nervous conductors. There is no good reason to think that effects are diffused from irradiated areas by nervous means alone. The "spontaneous" signals that are found with irradiated receptors, and the way these signals vary in size in response to stimuli, are better referred to changes in excitability in the receptors than to pathological signals.

The published evidence that nervous conductors show a stable change in excitability when nerve centres are irradiated [7, 8] shows that stable functional changes, that appear primarily in the excitability, propagate along nerve structures from the irradiated areas.

Professor Kirzon has used Vvedenskii and Utokhomskii's theory of parabiosis and of slowly changing processes in nerves to suppose that the more or less extensive damage sites, caused in nerves by irradiation, act as sources of the long-lasting effects that propagate to great distances along nerves. We have already given the main facts that confirm this idea [1]. We irradiated small (8–9 mm) areas of frogs' nerves and produced long-term excitability changes all along the nerve trunk; we used nerve–muscle preparations (sciatic nerve and gastrocnemius muscle with myographic methods). The changes in the response to stimulation in a normal nerve area were of

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two-phase type; they indicated that these slowly varying effects spread to the nerve–muscle transfer apparatus. The work reported here extends these studies; electrophysiological methods have been used.

METHODS

We used either frog sciatic nerve and gastrocnemius muscle (as before [1]), or else isolated frog sciatic nerves. The preparations were held in a special humid chamber (Fig. 1). Small lengths (8–9 mm) of the nerves were X-irradiated. The nerves were in all cases dissected out with their spinal cord roots, since we could then record the action potentials when the motor roots were stimulated, and could establish how the nerve pulses were changed by their passing through the irradiated zone (these pulses are of course directly related to muscle contraction). The roots also formed a considerable extension to the nerve, which was of assistance to us in

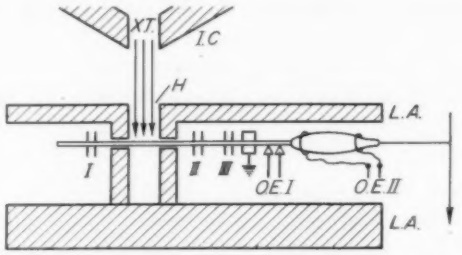

FIG. 1. Chamber and preparation. *X.T.*—X-ray tube; *I.C.*—iron collimator; *H*—hole; *L.A.*—lead and aluminium screen; *O.E.I* and *O.E.II*—outgoing electrodes; *I*, *II* and *III*—stimulator electrodes.

arranging the nerve in the chamber: the nerve had to be so coupled to four pairs of leads and to an earthed plate that the muscle did not contact the outgoing electrodes. Much of the nerve (13–14 mm) was concealed by the radiation screens or formed the irradiated area. As before [1], electrode pairs Nos. *I*, *II* and *III* were 10, 15 and 25 mm respectively from the irradiated area.

A square-wave generator [1] was used to stimulate the nerves; the pulse repetition rate was 50 sec^{-1}, and the pulse duration was from 0·7 to 1·0 msec (negative-going). Successive stimuli were separated by intervals of 30 sec. We recorded the contractions in the same way as before [1].

Silver electrodes of 0·5 mm diameter (outgoing electrodes *I*, Fig. 1) picked up the action potentials from the nerves; silver electrodes joined to the muscle via porous rods wetted with Ringer recorded the action potentials from the Achilles tendon and from the thickest part of the muscle (outgoing electrodes *II*, Fig. 1).

A double-beam oscilloscope with a type 2-OB photographic recorder was used to record the action potentials; this instrument was designed and made in the experimental workshops of the Academy of Medical Sciences of the U.S.S.R. in 1952.

The chamber was screened from external stray fields by an earthed iron box; all the leads were screened. Guliaev and Zhukov's method [9] was used, with an earthed platinum plate in front of the pick-up electrodes, to block out stimulus artifacts; also, the stimulating electrodes were joined to the source via a choke.

A URS-70 k-1 universal X-ray unit for use in spectral analysis acted as the X-ray source. The unfiltered emission at 30 kV and 14 mA was used, with the nerve in all cases 2 cm from the tube. The radiation dose was determined by the time (1 min ≡ 400–450 r), since the dose-rate was constant.

EXPERIMENTAL

First series

Isolated sciatic nerves were used; we recorded the action potentials caused by electrical stimuli applied to various parts of the nerve before, during and after irradiation, in all for 4–5 hr. Here the state of the nerve was found directly, and not indirectly as in [1], where muscle responses were used; the electrical activity of the nerve itself, which is closely related to the state and number of its elements, was used.

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We did the tests as follows. We recorded the action potentials caused by stimuli of various strengths applied to each of the three pairs of electrodes before the irradiation. The test stimuli were those that caused threshold, medium and strong responses (judged from the spike heights) before the irradiation. Any changes in excitability were established from the changed responses to these stimuli. The nerves were then irradiated for 20 min, a time chosen from our myographic tests [1] as one that gave the most marked changes in muscle contraction. We found that irradiating areas 8–9 mm long caused the spike heights to change; this was true when electrode *I* was stimulated, and the wave passed through the irradiated area, and when electrodes *II* and *III* were used, when the wave propagated in the normal areas only.

The changes were as follows. The action potentials given by electrodes *I* fell gradually, often to zero; those given by electrodes *II* and *III* were increased in height. A notable feature in the latter case was that this rise in height applied to spikes caused by threshold, medium and strong stimuli, although the latter stimuli had caused maximal responses prior to the irradiation. These changes appeared during the irradiation and continued to develop for 4–5 hr after the end. The changes in spike heights went with a parallel rise in the threshold for electrodes *I*, and a large fall in the thresholds for electrodes *II* and *III*.

Figure 2 shows typical results (from test No. 79); the spike heights given by electrodes *I*, *II* and *III* are shown. Those given by *I* fall slowly, while those given by *II* and *III* rise.

We thus find that irradiation changes the irradiated area (as is already known [10–12]), and also adjacent areas. The excitability is gradually and permanently raised in regions distal to the irradiated area, as the increased action potentials and lowered thresholds show. Our earlier experiments [1] showed that irradiating the nerve changed the response of the muscle to stimuli applied in normal areas of the nerve (Fig. 1, II and III). The changes in the muscle responses occurred in two phases; at first there was a rise above the original level in the responses to weak and medium stimuli, and a fall in the threshold (first phase). This was followed by a fall in the responses (even to zero), while the threshold remained very low relative to that found before the irradiation (second phase).

We supposed that the cause of the first phase was a rise in excitability in the screened areas of the nerve, so that more nerve fibres transmitted stimuli to the muscle, while the second phase had as cause some effect that propagated from

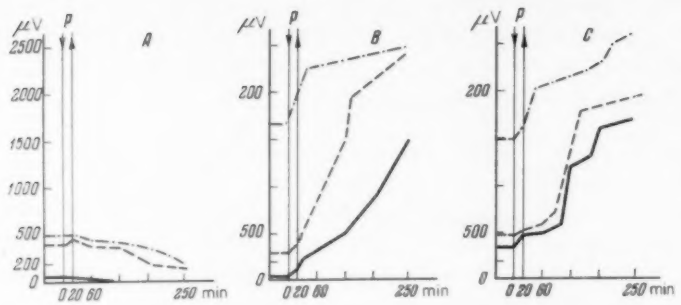

FIG. 2. Action potentials from a nerve elicited by electrodes in three screened areas (electrodes *I*, *II* and *III* of Fig. 1 correspond to sections *A*, *B* and *C*). Abscissa: time from start of irradiation. Ordinate: spike height, μV. The arrows *P* denote the start and finish of the irradiation. The full, broken and dot-and-dash lines correspond to threshold, medium and maximal stimuli respectively. *A*, *B* and *C*—graphs showing changes in the spike height of the nerve action potential produced as a result of nerve irritation with electrodes *I*, *II* and *III*. Continuous, dotted and dot–dash lines show the spike height of the nerve action potential produced as above. Threshold, average and maximum testing irritation points.

the irradiated area to the nerve–muscle synapses or even to the contractile areas of the muscle. The second effect (weakened responses) developed while the threshold remained low, so it is difficult to suppose that it was due to reduced excitability in the nerve.

The first set of tests confirmed our assumptions about the first phase, since the excitability was found to be increased. It was also found that this increased excitability found in shielded areas was long-lasting and rose with time. This confirms that the second phase is not due to lessened nerve excitability.

We did a second set of tests to check these deductions.

Second series

The nerve–muscle preparations of Fig. 1 were used. The nerve action potentials and the muscular contractions produced by electrodes *I*, *II* and *III* were recorded at the same time.

The tests were done as follows. The nerve action potentials and muscular contractions caused by electrical stimuli of various strengths were recorded before the irradiation. Three different strengths of stimuli were chosen for each pair of electrodes, to give threshold, medium and maximal responses from the muscle (the test stimuli). The nerve was then irradiated for 20 min. Fig. 3 shows the results for several members of this series, as the muscle responses and the action potentials plotted against time for test stimuli applied 25 mm from the irradiated section (Fig. 1, *III*). No diagram is given for electrodes *II*, since it is the same as for electrodes *III*. The black columns show the muscle's responses, the cross-hatched ones the action potentials from the nerve. The background level is that of the pre-irradiation responses and potentials.

The diagrams have been drawn up as follows. The absolute values for each test (cm for the muscular contractions, μV for the nerve potentials) have been converted

to per cent; the initial maximal contractions and maximal action potentials in the system before irradiation were taken as 100 per cent. The mean values were calculated for each point in a set, and the diagrams were constructed from the means.

Figure 3 shows that the action potentials and contractions began to increase as soon as the irradiation started, but that the two increases ran parallel only at the start (first phase), and then only for the threshold and medium stimuli (Fig. 3*A* and *B*). The action potentials continued to rise, but the contractions began to get smaller (second phase); the effect was especially well seen with the maximal stimuli (Fig. 3*C*).

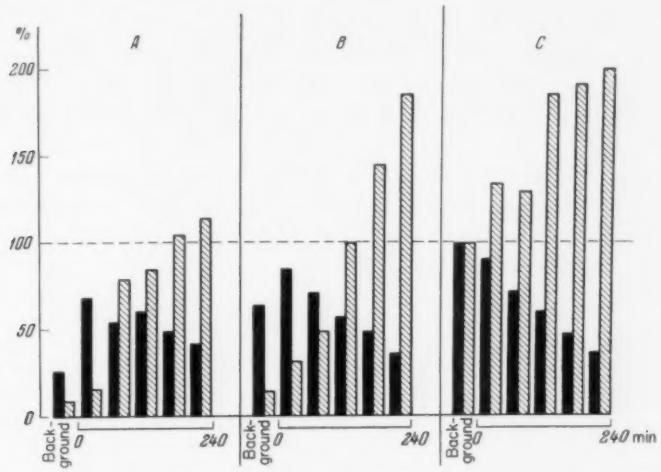

FIG. 3. Muscle contractions and nerve action potentials recorded simultaneously from stimuli applied to screened distal areas of nerve (electrodes *III* of Fig. 1). *A*, *B* and *C* correspond to threshold, medium and maximal stimuli respectively; all other symbols are explained in the text.

These data thus confirm our suppositions that the first phase is caused by increased nerve excitability, and that the second is caused by some change in the muscle itself or in the neuro-muscular junctions.

There are many signs [13, 14] that the neuro-muscular junctions are functionally the least stable part of the muscular apparatus, so we supposed that these junctions show the greatest changes produced by irradiating distal areas. The changes block the incoming nerve signals and thus weaken the muscle's response, i.e. cause the second phase found in the muscular contractions. We did a third set of tests to check this idea; we recorded the action potentials from the muscle, as well as those from the nerve, at the same time as the muscular contractions. We could then detect any functional changes in the junctions and could study the excitation process in the muscle.

Third series

We used the nerve–muscle preparation of Fig. 1 with a three-channel recording system for use with nerve–muscle preparations that has been used before [15]. The muscular contractions were recorded myographically, the action potentials from nerve

and muscle, oscillographically. The tests were done in the same sequence as in the previous set, except that action potentials were recorded from the muscle as well (Fig. 1, outgoing electrodes *II*).

The results showed that irradiation changed the muscle's action potentials; those potentials recorded when the nerve was stimulated via electrodes *I* decreased gradually throughout the experiment, as did the action potentials of the nerve. Those potentials that we recorded from the muscle when the nerve was stimulated via electrodes *II* and *III* showed two phases, as did the muscular contractions. The spikes increased in the first phase and decreased in the second. The second phase developed although the action potentials of the nerve continued to increase.

We thus have again that the second phase in the contractile responses produced by irradiation was of an independent nature, i.e. was caused by direct effects produced in the muscle by some influence that propagates along the nerve trunk from the irradiated area.

Figure 4 shows the data from test No. 105 in this series; it shows the muscular responses (*A*), the action potentials of the nerve (*B*) and those of the muscle (*C*), all of which were recorded together when the nerve was stimulated in a distal area 25 mm from the irradiated area (Fig. 1, *III*).

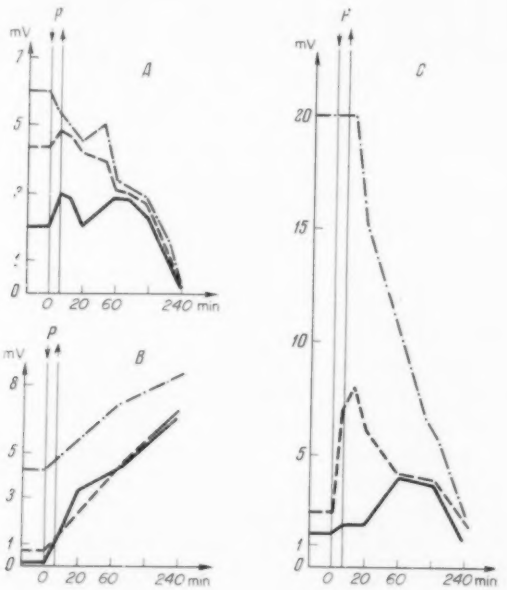

FIG. 4. Muscle action potentials, muscle contractions and nerve action potentials, recorded simultaneously from stimuli applied to screened distal areas of nerve (electrodes *III* of Fig. 1). *A* relates to the contractions, *B* to the nerve potentials and *C* to the muscle potentials. Abscissa: time from the start of irradiation. Ordinates: contractions (cm), or spike heights (mV) as appropriate. The arrows *P* denote the start and finish of the irradiation. The full, broken and dot-and-dash lines correspond to threshold, medium and maximal stimuli respectively.

We found no contractions, fibrillations, or nerve signals before the stimuli were applied, within the limits of the sensitivity of the oscillograph (13–15 μV), which limits were much lower than the threshold action potentials; this shows that no signals arise spontaneously from the irradiated area.

The results confirm our previous supposition [1] that local irradiation causes some functional effect to travel non-impulsively along the entire nerve; the effect is one of a stable change in excitability in the screened areas of the nerve. The two phases that we find on stimulating the nerve in an area distal to the irradiated area show that the effect spreads also to the muscle.

SUMMARY

(1) X-rays caused a rise in excitability that lasted for not less than 4–5 hr in distal areas of the nerve when they were allowed to act on 8–9 mm lengths of frog sciatic nerve. The action potential spikes from the nerve were heightened, whether the stimuli to the distal areas were threshold, medium or strong.

(2) This rise in excitability in distal areas went with two phases of change in the action potentials of grastrocnemius muscle, if the muscle potentials were recorded when the nerve was stimulated in distal areas. The muscle potentials at first rose and then fell. The contractile responses also showed two phases that ran parallel; some effect spread from the irradiated area to the muscle itself.

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THE ENZYMATIC BASIS OF TASTING

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Biochemical studies are of particular value in explaining the effects found in the taste and smell receptors; current work on this topic is concerned with the distributions of the enzyme systems within the peripheral organs, and with the effects of materials on these systems. Baradi and Bourne [1, 2] have used histochemical methods to demonstrate phosphatases, nucleotidases and lipases in man and mammals; these enzymes are variously affected in activity by quinine, saccharine, salts, etc. Landgren, Liljestrand and Zotterman [3] have shown that cholinesterase and the functionally related acetylcholine are also important to the sense of taste.

The problem can be studied biochemically in order to relate the chemical nature of the stimulus to the nervous activity it excites; Iureva [4] has recorded the currents in afferent taste nerve fibres and has shown that the sensitivity of the receptors is much reduced when the sulphydryl groups of a perfused tongue are blocked, and that liberating the groups with a unithiol restores the sensitivity. Iureva has demonstrated this effect even with tap and distilled water with frogs' tongues; the receptors are sensitive to osmotic pressure. The sensitivity to sugar is lost in the fly when the sulphydryl groups are blocked [5, 6]. This effect confirms Koshtoiants's idea that reactive groups in proteins are important to the sense of taste [6]; the sulphydryl groups determine many of the important steps in biological processes, so the relation of the sense of taste to the more important enzyme systems needs examination.

The relation of the sense of taste to the Krebs cycle is considered here, since this cycle is mainly responsible for the energy-rich compounds on which all functional activities depend [7]. We used the fact that malonate slows down the Krebs cycle but not if fumarate is present; the malonate–fumarate competition also has its analogies in physiological processes. Division of echinoderm eggs is depressed by malonate, but not if fumarate is present [7]; the depressed respiration caused in erythrocytes by malonate is much relieved by fumarate [9]. The same fumarate–malonate relation is found in the effects of potassium on brain-slice respiration [10] and on the contractile and electrical activities of the heart [11]; in both cases malonate depresses the effects of potassium, but not if fumarate is present.

METHODS AND RESULTS

Iureva's methods [4] were used with perfused frogs' tongues; the stimulants were 5 per cent solutions of glucose and of sodium chloride, and tap and distilled water, which also act as stimulants [12]. The stimulants were dropped on to the dorsal surface of the tongue from pipettes; oscillographic recordings were made from

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FIG. 1. (*A*) Pulses from *R. lingualis n. glossopharyngei* on treating the tongue with tap water (*I*) and 5 per cent glucose solution (*II*); (*B*) the same after adding 1·5 ml of 7×10^{-2} malonate to the perfusing Ringer; (*C*) as (*B*) but with 1 ml of malonate plus 1 ml of fumarate (10^{-2}); (*D*) the same on a background of Ringer perfusion 10 min after use of the malonate–fumarate mixture; bottom, background from Ringer alone.

FIG. 2. (*A*) Pulses from *R. lingualis n. glossopharyngei* on treating the tongue with distilled water (*I*) and 5 per cent sodium chloride solution (*II*); (*B*) the same after adding 2 ml of 7×10^{-2} malonate to the Ringer; (*C*) as (*B*), but with 1 ml of malonate plus 1 ml of succinate (7×10^{-2}); (*D*) the same on a background of Ringer perfusion 10 min after use of the malonate succinate mixture; bottom, background from Ringer alone.

Rana lingualis n. glossopharyngei. The sodium malonate was used at a concentration of 7×10^{-3} and 7×10^{-2}, the sodium fumarate at 10^{-2}, and the sodium succinate at 7×10^{-3}. All four stimulants gave highly abnormal taste signals when the tongue had been treated with malonate; the amplitudes and frequencies of the signals were much reduced (even to near background) (Figs. 1*B* and 2*B*). The activities became normal when fumarate or succinate was introduced into the perfusion system (effect of malonate on the Krebs cycle removed) (Figs. 1*A* and 2*A*). Malonate applied at the same time as fumarate (Fig. 1*C*) or succinate (Fig. 2*C*) gave a normal pattern with all the stimulants; the signals were even somewhat intensified on washing the tongue with Ringer with the fumarate (Fig. 1*D*) and succinate (Fig. 2*D*), particularly in the latter case, where the response occasionally exceeded the normal by two or three times.

These data confirm that excitation is enzymic in a general way; the chemical agents liberated by nervous means or by external stimulation cause excitation by their participation in enzymatic processes responsible for functional activity (and excitability) [12]. The precise detailed mechanisms of taste perception and of the related nervous activity require further examination.

SUMMARY

(1) Perfused frogs' tongues have been used to record oscillographically the activity in the afferent nerve produced by applying glucose, sodium chloride, and tap and distilled waters.

(2) The activity found in *R. lingualis n. glossopharyngei* was reduced or eliminated when malonate was applied; succinate and fumarate prevented malonate from having this effect.

(3) The responses to chemical stimuli depended very much on the state of the Krebs cycle, since this is one of the most important parts of tissue metabolism.

Translated by J. E. S. BRADLEY

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* There is an English translation of this work.

DEPENDENCE OF THE CRITICAL INTERVAL FOR DISTINCT COLOUR VISION IN MAN ON THE STRENGTH, DURATION AND SPATIAL DISTRIBUTION OF THE STIMULUS*

(THE CHRONOTOPE AND THE FUNCTIONAL VARIABILITY OF COLOUR VISION)

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THE main phenomena, such as excitability, functional lability, refractivity, reactivity, etc., which are encountered in the visual analyser are now considered to be related. The excitability is determined by the duration and spatial distribution of the stimulus, as well as by the threshold stimulus required. Lapique's theory of chronaxy takes account only of the stimulus duration; it has been extended and revised in chronotope theory [1, 3]. The chronotope factors determine the excitability according to the formula

$$E_R = 1/f(i, t, q) \tag{1}$$

where E_R is the excitability for some reaction R, i is the stimulus intensity, t is the time it acts and q is the irritable area.

Chronotope theory indicates that a function $f_1(i, t, q)$ exists for any system which is such that the excitability is maximal. Equation (1) then becomes

$$A_R = 1/f_1(i, t, q) \tag{2}$$

where A is the maximum excitability for the reaction R. A is termed "adequate" [1, 2]. Our problem is to determine how the critical interval of the visual analyser depends on the above quantities (colour stimuli). Several distinct colour stimuli following one another within a very short time are seen as one mixed stimulus, and not separately. The minimum time separation required for two stimuli to be seen separately is the critical interval [3]. This critical interval depends on the intensity and duration when achromatic stimuli are used; we have examined the relation for chromatic stimuli, and have sought to find whether the critical interval depends on how the stimulus is distributed over the retina (which it does). Our formula, using the symbols above, is $\tau = f(i, t, q)$, where f is some function.

Central vision stimuli and of wavelengths 620 mμ and 500–565 mμ (red and green) were used; i was measured in rheobase terms, t in msec and q (retinal area) in square millimetres. The subjects had normal vision and were aged 20 to 25 years.

METHODS

Makarov's optical "adequatometer" [2] was used; i, t and the spatial distribution could be varied. The apparatus has been fully described elsewhere [1, 3].

* Biofizika **3**: No. 6, 693–697, 1958.

It is difficult to determine how τ depends on all three variables simultaneously, so two were fixed at any one time. The variables were (a) i, with t and q constant, (b) t, with i and q constant, and (c) q, with i and t constant. Dark adaptation for 30–40 min before the tests was standard.

RESULTS

The most typical results for light of wave-length 620–690 mμ are given below; subscript zero denotes the minimum values used. Two of these minimum values were kept constant in any one test (Figs. 1–3; Tables 1–3); Table 1 relates to q_0 and t_0 constant, with $i > i_0$, and τ_0 is the value of τ found at $i = i_0$.

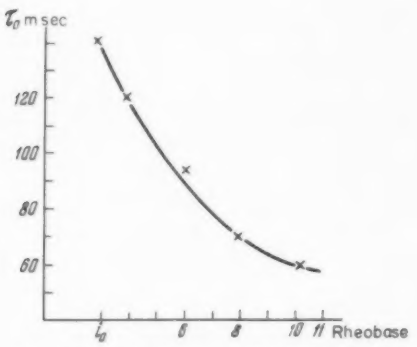

FIG. 1. Graph showing relationship between the critical interval and the light stimulus. Abscissa: intensity of light in rheobase. Ordinate: critical interval in milliseconds. τ_0 vs. i (in rheobase).

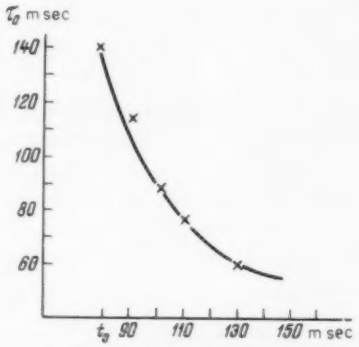

FIG. 2. Graph showing the relationship between the critical interval and the duration of the light stimulus. Abscissa: intensity of light in rheobase. Ordinate: critical interval in milliseconds. τ_0 vs. t (in msec).

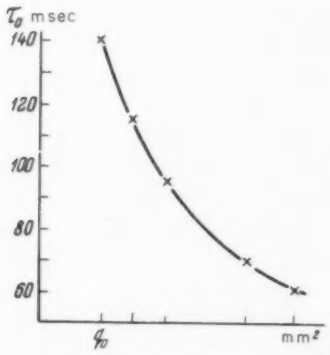

FIG. 3. Dependence of the critical interval on the size of the area irritated. Abscissa: area of visual disk (square millimetres). Ordinate: critical interval in milliseconds. τ_0 vs. q (in mm²).

TABLE 1. RESULTS FROM RED STIMULI

Time	q_0	t_0	i_0	τ_0	i	τ	$\tau_0 - \tau$
11.35 (start)							
12.20	78·5	60	3	140	—	—	—
12.25	78·5	60	—	—	4	120	20
12.30	78·5	60	—	—	6	95	45
12.35	78·5	60	—	—	8	70	70
12.40	78·5	60	—	—	10	60	80

TABLE 2. RESULTS FROM RED STIMULI

Time	q_0	i_0	t_0	τ_0	t	τ	$\tau_0 - \tau$
11.30 (start)							
12.15	78·5	2	80	140	—	—	—
12.20	78·5	2	—	—	90	115	25
12.25	78·5	2	—	—	100	90	50
12.30	78·5	2	—	—	110	75	65
12.35	78·5	2	—	—	130	60	80

TABLE 3. RESULTS FROM RED STIMULI

Time	i_0	t_0	q_0	τ_0	q	τ	$\tau_0 - \tau$
11.20 (start)							
11.50	3	80	12·56	140	—	—	—
11.55	3	80	—	—	29·26	115	25
12.00	3	80	—	—	50·24	95	45
12.05	3	80	—	—	78·5	70	70
12.10	3	80	—	—	113·04	60	80

The curves for 500–565 mμ stimuli are similar.

DISCUSSION

The results show that τ depends on i, q and t; we can describe the results by the differential equations

$$\frac{di}{i} = -c_1 d\tau,\ q = \text{const},\ t = \text{const}; \tag{3}$$

$$\frac{dt}{t} = -c_2 d\tau,\ i = \text{const},\ q = \text{const}; \tag{4}$$

$$\frac{dq}{q} = -c_3 d\tau,\ i = \text{const},\ t = \text{const}. \tag{5}$$

τ_0 becomes τ_n when i changes; this is true of all three equations. Integrating (3), (4) and (5) between suitable limits we have

$$\tau_{0,1} - \tau_{n,1} = \frac{1}{c_1} \ln \frac{i}{i_0}; \tag{6}$$

$$\tau_{0,2} - \tau_{n,2} = \frac{1}{c_2} \ln \frac{t}{t_0}; \tag{7}$$

$$\tau_{0,3} - \tau_{n,3} = \frac{1}{c_3} \ln \frac{q}{q_0}. \tag{8}$$

Let

$$\tau_{0,1} - \tau_{n,1} = \tau_1;$$
$$\tau_{0,2} - \tau_{n,2} = \tau_2;$$
$$\tau_{0,3} - \tau_{n,3} = \tau_3.$$

Then (6)–(8) become

$$\tau_1 = \frac{1}{c_1} \ln \frac{i}{i_0}; \tag{9}$$

$$\tau_2 = \frac{1}{c_2} \ln \frac{t}{t_0}; \tag{10}$$

$$\tau_3 = \frac{1}{c_3} \ln \frac{q}{q_0}. \tag{11}$$

Here τ_1, τ_2 and τ_3 are the differences from τ_0 found on increasing one of i_0, q_0 and t_0 while the other two remain fixed. The cs are of dimensions sec^{-1} and

$$c_1 = \frac{\ln \frac{i}{i_0}}{\tau_1}. \tag{12}$$

Equation (12) shows that c_1 indicates how much i must change to alter τ by 1 msec. Experiment gives us a mean c_1; Fig. 4 is the τ_1 curve constructed from Table 1 by calculation from (9) (shown broken). If $\lambda = 620$–690 mμ, $c_1 = 0{\cdot}015$, $c_2 = 0{\cdot}01$ and $c_3 = 0{\cdot}037$,

i	τ_1
4	14
6	45
8	65
10	75

The experimental and theoretical curves agree within the experimental error; the same applies to the curves constructed from (10) and (11). We can therefore construct curves in agreement with experiment which show how τ depends on i, t and q if c_1, c_2 and c_3 are known.

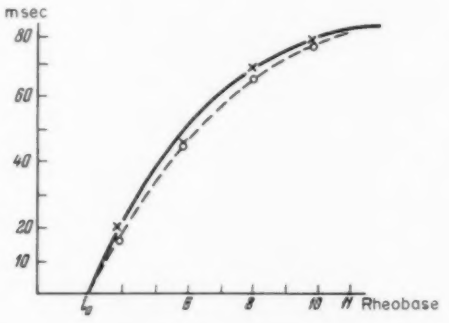

FIG. 4. Graph showing the dependence of increase in the critical interval on changes in the intensity of the stimulus. Abscissa: intensity of light stimulus in rheobase. Ordinate: increase in the critical interval in milliseconds. τ vs. i (in rheobase).

SUMMARY

(1) Foveal and parafoveal stimuli of wave-lengths 620–690 mμ and 500–565 mμ are used to show that the critical interval for discrete vision depends on the intensity, duration and spatial distribution (retinal area required).

(2) The experimental and theoretical results agree in the main.

Translated by J. E. S. BRADLEY

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CAROTENOID SYNTHESIS AND ACCUMULATION AS FUNCTIONS OF LIGHT INTENSITY AND SPECTRAL COMPOSITION*

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THE structures, biosyntheses, accumulation and relationships of the carotenoids have been reviewed [1], but the connections with chloroplast chlorophyll have scarcely been touched on. The carotenoids can be formed in the dark; the way the wave-length of the light may influence the syntheses is not clear, and even Goodwin [1, 2] gives no data. Shlyk [3] has used labelled glucose, acetic acid and glycerol; the precursor of the carotenoids and of phytol is found to be glucose. This confirms Godnev's idea [4, 5] that the syntheses of the carotenoids and of chlorophyll have a common basis in that of phytol. Frank [6] has shown that the absorption spectrum of porphyrin and the action spectra of carotenoid destruction and chlorophyll synthesis found at early stages in etiolated oat seedlings are identical. Granick [7] has found that in a *Chlorella* mutant grown at room temperature, the carotin is lost as the magnesium vinyl pheoporphyrin is transformed to protochlorophyll.

Wolken and Mellon [8] have shown that in *Euglena gracilis* the spectra required to produce chlorophyll and the carotenoids are the same; they conclude that some porphyrin-like substances can influence the syntheses.

It has been found that blue light favours carotenoid synthesis in oat seedlings [9] and in *Elodea* [10], while red light retards it. Chernavina [11] also concludes that blue light favours carotenoid synthesis in young plants. Wheat [12] and lettuce [13] show more carotenoid synthesis if grown in red light; this can be explained [14] as

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the precursors absorbing light (or even the carotenoids themselves) and being activated thereby. Light has no direct action on carotenoid synthesis [15, 16]; Claes concludes [17] that carotin can be synthesized in *Chlorella* either enzymatically or directly under irradiation.

METHODS AND RESULTS

Radishes, lettuce, cucumbers, *Vicia faba*, maize and wheat (spring and winter) were used at different ages; the pigments were assayed by Godnev's method [18], using a photoelectric colorimeter with extracts. The blue and red light sources used were fluorescent tubes or filament lamps fitted with liquid or gelatin filters; the methods have already been described in detail [19]. Tables 1 and 2, and Figs. 1 and 2, give the results; these show that up to 25 W/m² the plants grown in red light

TABLE 1. CAROTENOID CONTENTS OF PLANT LEAVES GROWN IN RED OR BLUE LIGHTS OF VARIOUS INTENSITIES IN mg/g DRY WEIGHT*

Wheat		Intensity (W/m²)							
		18–20		25		32–34		41–45	
		Blue	Red	Blue	Red	Blue	Red	Blue	Red
Winter	Lutescens 329	0·200	0·314	—	—	0·758	0·471	—	—
		0·314	0·392	—	—	0·906	0·700	—	—
		0·402	0·550	—	—	—	—	—	—
		0·502	0·530	—	—	—	—	—	—
		0·497	0·662	—	—	—	—	—	—
Winter	Koopera-torka	—	—	0·416	0·474	—	—	0·558	0·514
		—	—	0·420	0·504	—	—	0·578	0·477
		—	—	0·512	0·567	—	—	0·610	0·566
		—	—	0·521	0·557	—	—	0·620	0·456
		—	—	—	—	—	—	0·772	0·580
Spring	Lutescens 0·62	0·294	0·332	—	—	0·600	0·594	—	—
		0·377	0·516	—	—	0·826	0·790	—	—
		0·426	0·498	—	—	0·855	0·792	—	—
		0·437	0·631	—	—	—	—	—	—

* The results are arranged in order of increasing carotenoid content for convenience.

TABLE 2. CAROTENOID CONTENTS OF PLANT LEAVES GROWN IN RED OR BLUE LIGHTS OF HIGH INTENSITIES IN mg/g DRY WEIGHT

Plant	Intensity (W/m²)			
	40–45		60	
	Blue	Red	Blue	Red
Vicia faba	0·678	0·455	0·638	0·505
Maize	—	—	0·892	0·536
Cucumber	0·802	0·568	—	—
Radish	—	—	0·636	0·544
Lettuce	—	—	0·760	0·560

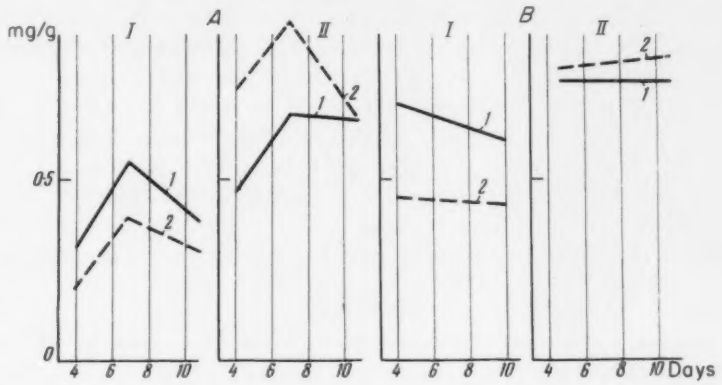

FIG. 1. Carotenoid contents of leaves from winter wheat (*A*) and spring wheat (*B*) grown in (1) red light, (2) blue light. *I*, intensity 18 W/m² of physiologically useful radiation; *II*, 3·3 W/m². Ordinate: content of carotenoid (mg/g, wet weight). Abscissa: number of days from emergence of shoots.

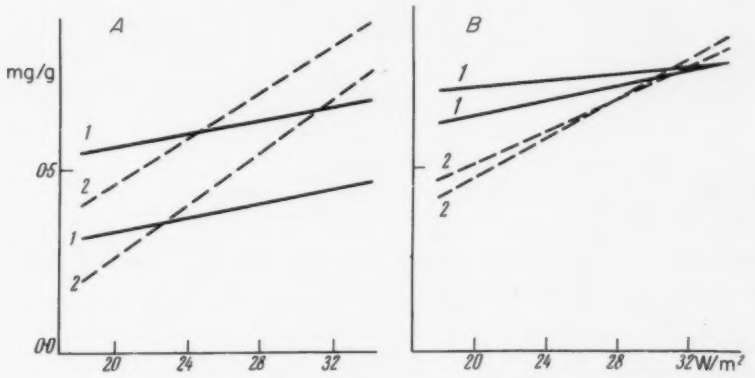

FIG. 2. Carotenoid contents of leaves from winter wheat (*A*) and spring wheat (*B*) grown in (1) red light, (2) blue light of different intensities. Ordinate: content of carotenoid (mg/g, dry weight). Abscissa: intensity W/m² of physiologically useful radiation.

show more carotenoids, but that those grown in blue show more at high intensities. The fluctuations are less in the former case (0·314 to 0·792 mg/g), although the intensity range (18–60 W/m²) is large; the fluctuations are from 0·200 to 0·906 mg/g and run parallel to the light intensity in the latter case (Fig. 2).

The largest amounts were found after 5–7 days; the values thereafter dropped. The effects of light intensity and colour are the same as have been found with chlorophyll [19], i.e. at low intensities the plants grown in red light show more carotenoids, but that those grown in blue show more at high intensities. The cause is the genetic relation between the pigments. The small fluctuations in content (independent of the intensity) found with the red light (which is not absorbed by the carotenoids) must mean that the carotenoids are synthesized mainly enzymatically.

The even smaller amounts found with low-intensity blue light may occur because inadequate amounts of precursors are formed. Red light is maximally absorbed by chlorophyll, so low intensities can provide adequate amounts of precursors. The correlation with intensity found with blue light is partly due to more precursor synthesis and partly to autocatalysis by the carotenoids themselves, which absorb blue light [14]. The rate-limiting step in red light is the maximum possible rate of enzymatic synthesis, which is reached at low intensities. We therefore suppose that the higher efficiency of high-intensity blue light in producing carotenoids which go to form phytol (a component of chlorophyll) will result in more chlorophyll synthesis [19].

SUMMARY

(1) Low-intensity blue light limits carotenoid synthesis because too small amounts of precursors are photosynthesized.

(2) High-intensity blue light produces much larger amounts of carotenoids because (*a*) large amounts of precursors are formed, roughly in proportion to the intensity, (*b*) the carotenoids absorb maximally in the blue region.

(3) In red light, which the carotenoids do not absorb, the carotenoids are formed mainly enzymatically; the enzymatic process is the rate-limiting step.

(4) The general resemblances between the effects of red and blue lights on carotenoid and chlorophyll syntheses [19] indicate that the two syntheses are closely related.

I am indebted to S. V. Tageeva for guidance and assistance in this work.

Translated by J. E. S. BRADLEY

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* There exists a translation in English of this work.

SOME REGULARITIES IN THE POST-IRRADIATION CHANGES IN RESTING YEAST CELLS*

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WE have previously shown [1] that γ-irradiated yeast cells given doses in the lethal range are not inactivated until a certain time has passed, i.e. until after a certain number of division cycles. We now examine how the post-irradiation changes are related to division, and whether the lower division rate is merely a passive reflection of the fact that the after-effects have reached some threshold value, or whether division itself plays an active part in causing the after-effects.

1. STAINING WITH NEUTRAL DYES AND SPONTANEOUS LYSIS

Introduction

One may judge whether a cell has died from how it takes up neutral dyes [2], or from its lysis. Staining reveals only those dead cells that have not lysed. So far as we are aware, staining and spontaneous lysis in unicellular organisms have not been studied in detail. There are only isolated statements that X-irradiated yeast cells immediately after irradiation stain no differently from the way that controls do [3], and that irradiated bacterial cultures lyse somewhat more rapidly than do control ones [3].

Methods

The yeast cells were brought to a resting state by keeping them in sterile tap water or on neutral agar. A GUT-Co-400 unit was the radiation source, which gave dose-rates of 1020 r/min; the exposure times were varied. We used, as before [1], *Saccharomyces vini* (Megri-139-B strain), with suspensions of the resting cells (48 hr culture on wort agar slopes at 30°) with 6×10^5 cells per millilitre.

We tested several dyes (methylene blue, neutral red, gentian violet, etc.) and finally chose erythrosin, which Professor Khudiakov recommends; in 0·1% aqueous solution it rapidly stains dead cells a bright purple. The control and irradiated cells were incubated as required on a non-nutritive medium at 30° before they were stained for 30 min. The stained forms were counted in batches of 1000 cells.

The spontaneous lysis rates were recorded at the same time. The cells were spread out on slides coated with 2% agar in tap water and were incubated in a humid chamber at 30°. The numbers of normal cells were counted at ×200 at fixed points in the preparations at once, and every 24–48 hr after the irradiation. Some 1200 to 1700 cells were under observation in the slides. Live cells were seen as convex glistening globules; dead cells were seen as dark flat shapes, before the cell wall and

* Biofizika **3**: No. 6, 703–710, 1958.

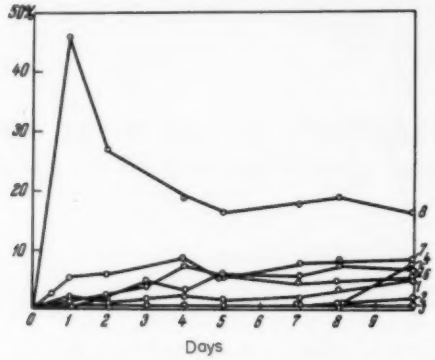

FIG. 1. Stained-form contents of irradiated yeast cell suspensions. (1) Control, (2) 10 kr, (3) 20 kr, (4) 40 kr, (5) 80 kr, (6) 160 kr, (7) 280 kr, (8) 500 kr.

central areas began to break down. Typical haloes were seen in the agar round such cells, which were surrounded by small granules that finally broke up and vanished.

The staining and spontaneous lysis rates were examined during 10 day incubations that followed various doses; two parallel sets were run. The results usually agreed within 10–15 per cent.

RESULTS

Figure 1 shows that suspensions that had been given doses of 10–20 kr showed stained-form contents somewhat below those that the controls did at all times. The difference rose to 6–8 per cent at 3–4 days with doses of 100–300 kr (i.e. exceeded the control somewhat) and thereafter stayed at the same level. The pattern given by 500 kr doses was different. The stained-form content rose rapidly in the first hours after irradiation and reached 46–52 per cent after a day; it then began to fall (because the dead cells lysed), and fell to 18–20 per cent at 4 days, at which level it thereafter stayed.

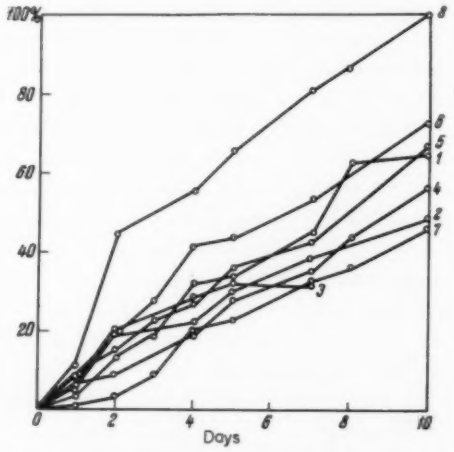

FIG. 2. Spontaneous lysis curves for irradiated yeast cells. (1) Control, (2) 10 kr, (3) 20 kr, (4) 40 kr, (5) 80 kr, (6) 160 kr, (7) 280 kr, (8) 500 kr.

The data show that low doses caused the death rate in a resting culture to rise little over the rates found in controls. Doses of 100 kr caused some of the cells to die rapidly (within a day), while the surviving cells showed a rate of spontaneous lysis little different from that shown by controls. The content of stained forms equalled the difference between the relative numbers of cells that had died and that had already lysed. The data thus more truly reflect the state of the cells that had survived than the rate of spontaneous lysis. Some tests with non-nutritive agar gave more direct data on the spontaneous lysis (Fig. 2). These data confirm our conception of the lysis process completely, when taken in conjunction with our erythrosin staining studies. The effects appeared somewhat more rapidly with agar, perhaps because aeration was better.

DISCUSSION

Doses of less than 300 kr of γ-rays had almost no effect on the spontaneous lysis rates shown by yeast; 500 kr caused about half of the cells to die and to lyse in the first 24–48 hr, and the other half of the cells to behave as if they had been irradiated at lower doses. Fixed microcolony methods [1] showed that the number of cells that died within 24 hr of irradiation was, within the errors of experiment, equal to the number that the irradiation had caused to lose the capacity to reproduce. Cells that could not divide even once after irradiation in general died while they were still in the beam, or immediately afterwards. Rajewsky has found that higher animals as well as micro-organisms die immediately after irradiation only at doses above 100 kr. Rajewsky considers that the type of death found at these high doses is of "molecular" kind, i.e. occurs because vital substances are destroyed [5].

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2. RECOVERY IN IRRADIATED RESTING YEAST CELLS

Introduction

Cells that have been given sub-lethal doses and have then been placed in a non-nutritive medium show no difference in death rate from that shown by control cells; this indicates that the damage produced by the irradiation can appear only when the cells metabolize. The latent damage may change in type (may become less or more serious) while the cells are kept in a resting state. The time that the cells stay in such a state should then affect the damage pattern that is seen when the cells are placed in optimal growth conditions.

Effects which can appear only via metabolism may be called primary damage. Our method of fixing microcolonies [1] is most suitable for use with immobilized unicellular organisms. The data it gives are the per cent inactivation (M) and the mean number of division cycles before death in irreversibly damaged cells (Q). If the primary damage is accentuated by a resting period, we should find that M increases and Q decreases; if it is lessened, Q should increase and M decrease; if some cells recover while others become more damaged, both M and Q should fall.

Methods

Vials containing irradiated and control suspensions were incubated in a thermostatic oven at 30° for 7 days in our tests on the effects of a resting period. The vials were sampled in a sterile fashion at set intervals and the samples were spread on nutritive agar on slides; our method of fixing microcolonies was used. The control

suspensions were ones that had been freshly made, or ones that had been kept on non-nutritive media for various times. The tests were done on two parallel sets.

Results

Figures 3 and 4 and Table 1 show the results; the time for which the cells are kept at 30° on a non-nutritive agar has no real effect on their radiosensitivity, while post-radiation incubation in the same way on a non-nutritive agar greatly increases the survival rate (Fig. 3). The effect cannot be caused by selective lysis of irreversibly

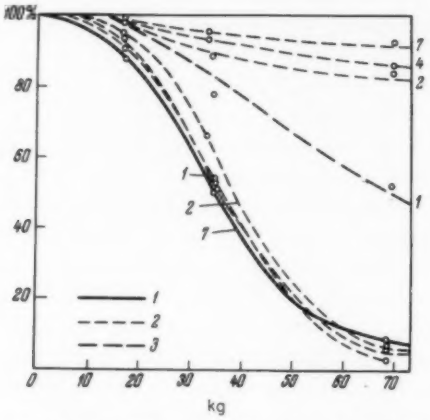

FIG. 3. The effects on the shapes of the survival curves of incubation on a non-nutritive medium before and after irradiation. (1) Standard survival curve, (2) incubation before irradiation, (3) incubation after irradiation. On right: incubation times in days.

inactivated individuals, since the lysis rates of cells that have been given doses of 10 r to 100 kr do not differ essentially from those of controls (see above), and very few cells lyse in the first day of incubation. The effect must be caused by spontaneous recovery from the damage received.

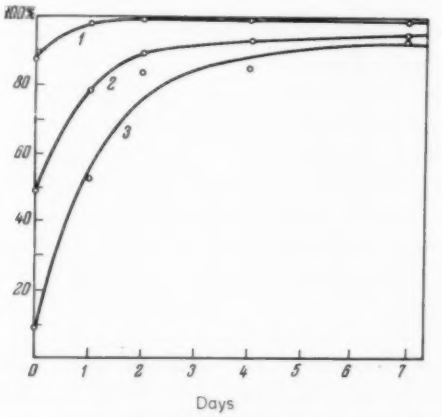

FIG. 4. Survival in irradiated yeast cells as functions of incubation time on a non-nutritive medium after irradiation. (1) 17 kr, (2) 34 kr, (3) 68 kr.

TABLE 1. THE EFFECTS OF INCUBATION AFTER IRRADIATION ON THE NUMBER OF DIVISION CYCLES BEFORE IRREVERSIBLE INACTIVATION (68 kr)

Incubation time (days)	0	1	2	4	7
Per cent survival	9±4	52±4	84±3	85±3	93±2
No. of cells in a colony, n	21±20	73±38	79±36	82±46	87±39
$Q = \log_2 n$	4·4	6·2	6·3	6·4	6·5

The data show the number of cycles prior to inactivation is also increased by incubation after irradiation; all the irradiated cells recovered, and incubation on a non-nutritive medium acted like a reduction of the radiation dose. Figure 4 shows how the per cent survival is related to this incubation time.

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DISCUSSION

Yeast cells could thus recover partly if kept on a non-nutritive medium after irradiation, even if the incubation medium was irradiated water. Long contact between irradiated cells and irradiated medium caused no real fall in viability at the doses that we used; no effect from the irradiated medium could be seen separate from the recovery effects.

The recovery may be caused either by toxic products' diffusing out of the cells, or by macromolecules' recovering spontaneously from damage; whatever the cause, recovery certainly occurs. Recovery may well go on during irradiation; only those cells are inactivated that have damage involving metabolic processes. It may well be that all types of organism would show radiosensitivities that depend on the times for which the organisms are kept in states of lowered metabolic activity after irradiation. It seems to us that this is quite likely, although little has been published on spontaneous recovery [6]. Chick embryo tissues that have been kept at 0° for 5 hr after irradiation show no radiation damage [7]; Polikarpov* has found like results with hydra. Pratt *et al.* [8] have examined the effect in more detail with *B. coli*; the irradiated cells were cooled to 4–6°, and the viability curve was determined in the standard way by sampling the culture daily for 6 days. The *B. coli* showed a recovery pattern very like that shown by our yeast cells. It can hardly be a coincidence that two organisms so different give similar results.

The spontaneous recovery effect has probably the same cause as has the dose-rate dependence that is found for radiosensitivities; Crowther [9] was the first to describe the latter effect, and Lea [10] the first to study it in detail. Lea tried to interpret the results mathematically and inserted a recovery factor β in Crowther's formula. Reboul's similar calculations [11] envisaged β as a factor that gave the probability that a given dose would be ineffective. The mechanisms that are responsible for dose-rate effects have not yet been elucidated; if spontaneous recovery is related to changes in the primary damage, we should be able to study this primary damage directly. One method would then be to use tissues and cells that are kept in states of low metabolic activity in media of various temperatures, oxygen contents, pressures, states of aggregation, etc., to establish the laws of recovery.

* Unpublished results; personal communication.

SUMMARY

(1) It has been shown how the spontaneous lysis rate, neutral dye uptake and damage are affected for yeast cells that have been irradiated with γ-rays and have then been kept on a non-nutritive medium.

(2) The times at which irradiated resting cells died did not differ essentially from those for controls when the radiation doses were only part-lethal. Absolutely lethal (500 kr) doses caused part of the cells to die and lyse rapidly; the fraction of the cells that did this was about equal to the fraction that is inactivated without division [1]. It is very probable that the processes that cause cells to be inactivated after a few division cycles cannot proceed far or at all if the cells are not metabolically active.

(3) Yeast cells recovered if they were kept at rest after γ-irradiation. The laws of such recovery may serve as one means of elucidating the nature of the primary damage.

Translated by J. E. S. BRADLEY

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* There is an English translation of this work.

CHANGES PRODUCED BY GAMMA-RAYS IN THE PHYSICO-CHEMICAL PROPERTIES OF ERYTHROCYTES*

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THE radioresistance shown by erythrocytes [1] is now common knowledge; however their radiosensitivity can be increased by altering the salt composition and pH [2]. Prehaemolytic changes, which never go through to haemolysis, are caused by moderate doses [3–5]. Little is yet known about the mechanism of radiation haemolysis and the stages which precede it, although much work has been done on irradiated erythrocytes. The changes in various properties of the cells are merely stated, and not compared one with another, in most cases. In particular, permeability and electrical

* Biofizika **3:** No. 6, 711–716, 1958.

conductivity studies are usually divorced from those on the submicroscopic structures. An attempt is made here to correlate the electrochemical changes with those seen in the electron microscope.

The electrical conductivity changes were studied because these directly reflect the haemolytic effects. As long ago as 1923 it was found [6] that the low-frequency resistance was much increased by hypotonic haemolysis, it was later shown [7] that the swelling (which removes some of the haemoglobin and ions while leaving the stroma intact) was responsible for the effect. Glucosides (saponin, digitonin) and bile and fatty acid salts, on the other hand, leave the resistance of the stroma almost unchanged, although the haematocrit indicates considerable swelling [8, 9]. The stromocytes do not lose all their haemoglobin in the latter case, although much of the loosely bound lipid is lost; they thus become better conductors at low frequencies [9].

More concentrated solutions of these haemolysers cause the low-frequency resistance of the erythrocytes to fall; the stroma disintegrates more or less completely (stromatolysis) [6, 7, 10]. The type and degree of damage can thus be established by measuring the electrical conductivity.

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MATERIALS AND METHODS

We used rat erythrocytes in 3·5 per cent sodium citrate, and human erythrocytes stabilized in sodium oxalate.* The erythrocytes were washed three times with physiological saline after removal of the plasma by centrifugation. The moist mass was irradiated at 6·5 cm from the source on a GUT-400 unit at a dose rate of 700 r/min; total doses of 42 and 84 kr were given. The cells were incubated for 2 hr after irradiation; the haemolysis which occurred, but which is normally missed at these doses, was thereby detected.

The conductivities were measured at 22°C at 1 and 10 kc/s on an equi-arm bridge made up from non-inductive resistance boxes; a M-91/A galvanometer was used as null detector. In certain cases a transformer output to an oscilloscope was used instead of the galvanometer.

An EM-100 electron microscope was used at $\times 10{,}000$ to record the electron micrographs; the preparations were shadowed at 15° with chromium.

RESULTS

Conductivity measurements were made every 30 min; the resistance increased for 6–8 hr after the irradiation plus incubation (42 kr); at 2 hr the resistance was 122–125 per cent of the initial value (Fig. 1). The result with 84 kr was different; there was an initial rise, followed by a fall, though the original level was not always reached (Fig. 1).

We may say [7] that the rise corresponds to stromocyte swelling; some of the haemoglobin and ions are lost, but the structure is otherwise intact. It is more difficult to interpret the second stage at 84 kr; stromatolysis can be ruled out, since the original resistance is not regained. It cannot be that the cells shrink; the increase in volume shown by a haemolysed erythrocyte is irreversible. We must suppose that the swollen cells conduct better, though not as well as in stromatolysis, where the resistance is only 1–2 per cent higher than that of the wash liquid [10].

* We are indebted to Professor B. A. Kudriashov for his gift of materials.

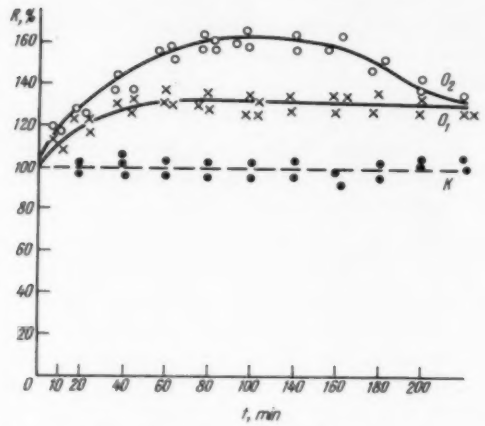

FIG. 1. Resistance changes in packed erythrocytes after irradiation. O_1, 42 kr; O_2, 84 kr; t, time after irradiation.

The stroma can conduct if the time-scales of ionic diffusion and electrical alternation are comparable [9]; the low-frequency conductivity appears when the lipid (of low dielectric constants) are lost. The second stage (reduction in resistance) found at 84 kr can thus be assigned to break-up of submicroscopic structures. Our results in the main confirm this view. At 42 kr no gross changes in the submicroscopic structures were found; the stroma remained circular in outline and flattened, with rare protrusions at the edges. Areas of differing densities are visible, and so is layering, as in hypotonic haemolysis. A distinctive feature of the radiation effects is that granules migrate to the edges; these may be coagulated areas of the surface (Fig. 2*a*). Lindemann [11] makes similar deductions about some analogous structures. The similarity between the two effects indicates that the irradiated erythrocytes would free more ions than do normal ones when acted on by some other agent as well. We used isotonic (8%) sucrose as this agent. No final conclusion has been reached on how irradiation affects permeabilities [12]; we therefore examined how well Lehman and Wels' early work [13]* compared with the results from using γ-rays.

The packed erythrocytes were washed three times with the 8% sucrose solution; 1 ml was then suspended in 1 or 2 ml of the solution. The mixture was carefully stirred and the cells recovered by centrifuging them down. The resistivities of the packed cells and of the supernatant were measured. The cells were not incubated in this series of tests, to avoid haemolysis, because the ionic yields in the prehaemolytic stages were of interest. The measurements were made at 15 min, 30 min, 1 hr and then every hour for 6 hr (Fig. 3). The γ-rays produce effects similar to those of X-rays. The increased ionic yields and resistivities of the irradiated cells suggested that hypotonic haemolysis and irradiation produce similar effects, but special experiments would be required to show that the two mechanisms were the same. If they were, the agents should act additively; haemolysed erythrocytes should show a higher resistivity if previously irradiated than if not. This effect was sought by disrupting irradiated and normal erythrocytes with distilled water (4 ml added drop by drop to

* On the increased yield of ions from X-irradiated erythrocytes.

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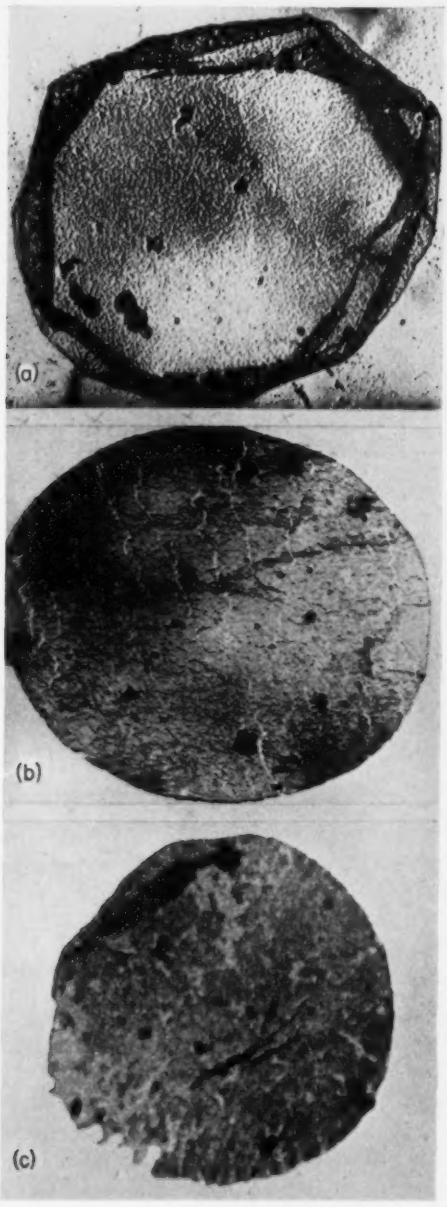

FIG. 2. Electron microscope pictures of erythrocyte stromas following the action of γ-radiation. (*a*) Apparent formation of folds while a rounded shape and an intact surface is maintained. (*b*) Dose of 84 kr; contraction of the structure and appearance of the surface. (*c*) Dose of 84 kr and following hypotonic haemolysis; destruction of the surface; formation of craters and holes.

1 ml of packed cells). Figure 4 shows that the irradiated cells (*O*) showed a slow and steady fall in resistivity, unlike the control cells (*K*). The effect resembles the stromoporosis first described by Petrova, who used high (over 0·01%) saponin concentrations; the cells are greatly damaged at the molecular level. The effect develops more slowly than does stromoporosis; the final resistivity of the cells is much higher than that of the liquid.

The conclusions from the resistivities are confirmed by the electron micrographs. Large pieces of the stroma were seen to have broken away in the haemolysed irradiated cells; fibrous structures and pits were seen. These pits were probably the sites of the

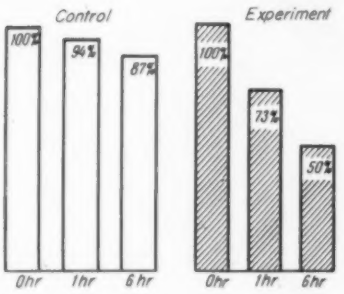

FIG. 3. Loss of ions from normal and irradiated erythrocytes (resistance values as percentages).

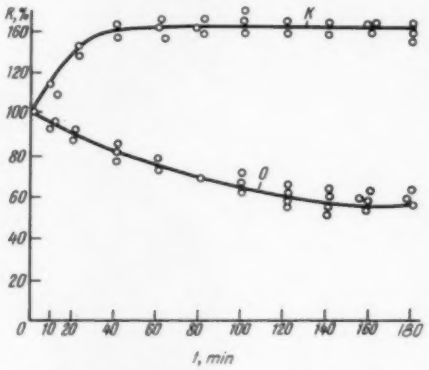

FIG. 4. Effects of hypertonicity on normal and irradiated erythrocytes. K = control.

loosely bound lipids that had been lost. Irradiation by itself causes no major damage to the submicroscopic structures, contrary to Zacek and Rosenberg's conclusions [14]. The γ-rays so alter the cells that they break up in hypotonic solutions, though.

The data show that Failla's hypothesis [15], in which radiation and hypotonic haemolysis mechanisms are equated, must be reconsidered. Radiation haemolysis occurs because the complex lipoprotein combinations responsible for the high resistivities of intact erythrocytes break up.

SUMMARY

(1) When 42 kr of γ-rays was delivered at 700 r/min to human erythrocytes which were then incubated, the low frequency resistivity increased and the stromocytes swelled while retaining their submicroscopic structures. Electron micrographs show no major changes in the stroma.

(2) A dose of 84 kr increased the low-frequency conductivity slightly and definitely disrupted the structures; electron micrographs confirm this.

(3) Irradiated erythrocytes (not incubated) free more ions into an electrolyte-free medium than do normal cells.

(4) Irradiated cells haemolysed with distilled water showed a gradual decrease in resistivity, which paralleled stromoporosis. The electron micrographs show pits, fibrous structures and pieces broken off; they confirm the deductions from the resistivities.

(5) The results cast doubt on Failla's hypothesis, in which radiation and hypotonic haemolysis mechanisms are equated. Radiation haemolysis occurs because the complex lipoprotein combinations responsible for the high resistivities of intact erythrocytes break up.

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EFFECTS OF X-RAYS AND EDTA ON CHROMOSOME BREAKS IN *TRADESCANTIA PALUDOSA* MICROSPORES*

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THE chromosome changes produced by ionizing radiations and radiomimetic substances can reveal how these agents act on the living cell; these agents can also be used to study chromosome structures. The microspores of *Tradescantia paludosa* were used in our studies on the combined effects of radiation and ethylenediaminetetra-acetic acid (EDTA) because these spores are cytologically very convenient. There is evidence that the number of chromosome crossovers is non-additively increased when these two agents act together [1], but the types of change produced have not been examined.

MATERIALS AND METHODS

Sax and his school have been much aided in their studies on radiation-induced chromosome changes by using *Tradescantia paludosa* microspores. Sax has isolated several clones which show virtually no spontaneous crossovers. Professor Gustafsson has kindly presented us with four plants from one such clone. The microspores from this Sax clone are very convenient for nuclear studies as the various phases of the division cycle last for long periods, the anther cells in a single bud develop in fair

* Biofizika **3:** No. 6, 717–724, 1958.

synchronism and the haploid nucleus consists of four large chromosomes in the first post-meiotic mitosis. The published data [2–5] show that the first post-meiotic mitosis lasts 7 days at $+30 \pm 0{\cdot}5$°C. Our work was done at $+19$ to $+21$°C. Table 1 gives the phase durations at this temperature. Ionizing radiations retard some phases, but the effect is slight at the doses we used (see [6, 7] on this question). In all cases we need 200 r doses (180 kV, 15 mA, 0·5 mm Cu filter, dose rate 41 r/min) at $+20$°C with uncut inflorescences on the plants. The EDTA concentration was varied (0·05 to 0·001 M), and so was the exposure time. The EDTA solution was injected continuously into the bud for 5 min in each half-hour over the period of a day, with a break of 10 hr during the night in long exposures.

TABLE 1. PHASE DURATIONS IN *Tradescantia paludosa* MICROSPORE DIVISIONS AT 19–21°C

Day	Phase duration		State of nucleus
	Hours	Minutes	
1–7	168	—	Interphase
8	36	—	Early prophase
9	5	—	Middle prophase
	4	—	Late prophase
	—	45	Metaphase
	—	45	Anaphase
	—	45	Telophase
10	24	—	Interphase in pollen grain

One or several inflorescences were used in each case. The buds in a single inflorescence were always in different stages; as the microspores reached metaphase the buds were removed and fixed. The fixation was therefore some time later than the treatment; the phase at which the spores were treated was calculated from the time elapsed. Thus a delay of 40 hr corresponded to early prophase treatment, 66 hr to late interphase, 120 hr to early interphase, etc.

The anthers were fixed in a 3:1 mixture of absolute alcohol and glacial acetic acid for 10–15 min, washed with 70 per cent alcohol and made up into temporary acetocarmine preparations.

All types of chromosome crossover, etc., found in the microspores were counted, i.e. fragments, acentric and centric rings and dicentric chromosomes. Inversions and translocations also usually occurred but were difficult to detect; they were therefore not recorded. Chromatid and isochromatid fragments, acentric and centric rings, dicentrics and exchanges were counted. Chromatid exchanges, or translocations, could be seen in metaphase only; the others could be recognized in anaphase as well. The semichromatid dicentrics were also counted when semichromatid changes occurred.

X-RAY EFFECTS

The microspores were irradiated 44, 60 and 120 hr before metaphase; the 44 hr (early prophase) irradiations caused chromatid changes, amongst others. The 60 and 120 hr irradiations produced only chromosome changes. Figure 1 shows how the

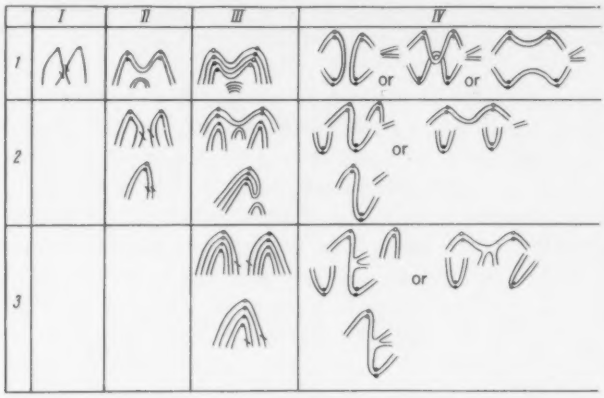

FIG. 1. Chromosome crossovers; formation of dicentrics. I, interphase; II, prophase; III, prometaphase; IV, anaphase; (1) chromosome, (2) chromatid and (3) semichromatid crossovers.

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dicentrics of chromosome, chromatid and semichromatid types were formed after irradiations at various phases. Early prophase is the moment when the chromosome is effectively split up, i.e. when either of its two components may break separately. The chromosome is shown as two threads in the figure at this stage. Under some conditions any of the four component semichromatids can break separately; the chromosome is then effectively split into four. The chromosome is then shown as four threads.

Ionizing radiation can produce chromosome breaks; the two fragments can change in three ways, namely, they may (1) join together again, (2) join up with other similar fragments, or (3) become incapable of rejoining ("sealed" fragments). The initial fragments may persist for some time in their original state; fractionated-dose and dose rate variation tests are directed to measuring this time. The values are found to range from seconds up even to months; the time undoubtedly depends on the state of the cell; e.g. the time is reduced in anoxia.

If the fragments formed in interphase were still capable of rejoining at the start

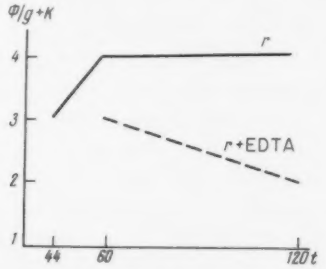

FIG. 2. Types of chromosome crossover produced in *Tradescantia paludosa* microspores by 200 r X-ray doses, following treatment with 0·001 M EDTA for 42 hr, or without EDTA. Ratio of fragments (φ) to dicentrics plus rings ($g+K$) vs. time (t) from irradiation to counting, in hours.

of prophase there would be no division in time between chromosome and chromatid rearrangements. A reasonably sharp boundary between the two effects is found, however.

All centric fragments and rings, and dicentrics, do not differ from normal chromosomes as regards the cycle in a single cell, whereas acentric fragments and rings are held up in development. An acentric ring fragment may be seen as a ring in a binucleate cell when the other chromosomes have already reached interphase, for instance. Table 2 gives the data on the changes produced by X-irradiation in early prophase and in early and late interphase. Taking the number in early interphase as unity, the ratios for late interphase and early prophase were 1·0: 1·1: 1·9. Interphase was least sensitive and prophase–anaphase the most, if chromosome effects were taken as index; the data on this topic are numerous [8–11], though Darlington's [12] *ex cathedra* assertion that interphase is the most sensitive is still accepted by some. Our data, and others, contradict this assertion.

The ratio of the fragments to the sum of the dicentrics and rings was three if prophase is irradiated, and four if interphase, so prophase produced more recombinations than interphase (see Fig. 2). The curled-up state of the chromosomes in prophase caused many recombinations, but this could not explain the higher radiosensitivity, since prophase gave more fragments than interphase (Table 2). The data at present available do not explain why the chromosomes vary in sensitivity during the cycle. The number of crossovers depends on how many fragments rejoin in the old way. The chromosomes themselves probably vary in sensitivity during the cycle; a given number of ionizations produces different numbers of primary breaks. Factors which may influence the number of crossovers are the energy balance of the cell, the mechanical curling in the chromosomes and the enzymatic activity of the cell.

THE EFFECTS OF EDTA

Mazia's work [13] has led some investigators [1, 14, 15] to try the effects of EDTA on the cell nucleus. Mazia himself concluded from his work on EDTA-induced chromosome fragmentation that the DNA and protein parts of the chromosomes are held together by Ca^{2+} and Mg^{2+} ions, and that the EDTA specifically extracts these cations. Kaufmann [16] has criticized this view; he considers that the EDTA alters the ion concentration in the cell, and does not extract cations from the chromosomes.

The time the EDTA acted on the microspores, and the EDTA concentration, were varied in our experiments. The cells were always completely killed by exposure to 0·01–0·05 M EDTA for 1 hr. If a 24 hr exposure to 0·002 M was begun at middle prophase the chromosomes stuck together; with a 42 hr exposure to 0·001 M starting in early prophase we found chromosome crossovers. The chromosomes in the latter case also lost part of their matrix material; the spiral structures could be clearly seen. In Fig. 3, cells (1), (2) and (3) show this well; 1·11 per cent crossovers occurred in this case and no centric or acentric rings, or chromatid exchanges, were seen. The ratio of fragments to dicentrics was 2·25, so many recombinations must have occurred.

The results when the EDTA (0·001 M) acted for 42 hr in interphase were analysed; no crossovers were found, though this may be because few chromosomes (480) were studied. Here the matrix was unaffected, although the chromosomes took up the carmine less readily than normally.

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TABLE 2. CHROMOSOME CHANGES IN *Tradescantia paludosa* PRODUCED BY 200 r OF X-RAYS AND 0·001 M EDTA

Previous EDTA treatment for 42 hr	Time from irradiation to count (hr)	No. of chromosomes examined	Crossovers		Fragments		Dicentrics		Centric rings		Acentric rings	
			No.	%	No.	%	No.	%	No.	%	No.	%
No	44	1326	128	9·6 ± 0·26	96	7·2	27	2·0	5	0·4	—	—
No	60	1650	91	5·5 ± 0·18	73	4·4	14	0·8	2	0·1	2	0·1
No	120	3432	175	5·1 ± 0·12	137	4·0	28	0·8	7	0·2	2	0·1
Yes	Not irradiated	1260	14	1·1 ± 0·29	10	0·8	4	0·3	—	—	—	—
Yes	62	1374	346	25·2 ± 1·17	260	18·9	71	5·2	11	0·8	4	0·3
Yes	120	1080	204	18·9 ± 1·18	123	11·4	68	6·3	9	0·8	4	0·4

FIG. 3. Types of chromosome crossover occurring in microspores of *Tradescantia paludosa* after exposure to 0·001 M EDTA for 42 hr and X-irradiation with a dose of 200 r. (1) Normal metaphase. (2) Dicentric chromosome and fragment observed 120 hr after treatment. (3) Chromosomal fragments acted on by EDTA. (4) Chromosome crossovers observed 36 hr after preliminary exposure to EDTA. (5, 6 and 10) Observed 62 hr after preliminary exposure to EDTA: dicentric chromosome, centric ring, and two fragments (5); dicentric chromosome, two chromosome fragments and one chromatid fragment (6); dicentric semichromatid, centric chromatid ring, and fragment (10). (7) Chromosomal bridge and fragments observed 120 hr after treatment. (8 and 9) Observed 120 hr after preliminary exposure to EDTA: chromatid bridge and fragments (8); chromosome bridge, chromatid bridge, two and one chromatid fragments (9).

It is clear that EDTA affected the matrix, and fragmented the chromosomes when appropriately applied. It may be that ionic bonds within the chromosomes were broken because the ion concentration in the cell is altered.

THE COMBINED ACTIONS OF EDTA AND X-RAYS

It has been shown [1] that X-rays and EDTA do not act additively on *Vicia faba* root-tips; Steffensen [15] has confirmed this, and has shown that too little calcium in the microspores causes chromosome crossovers, the numbers of which increase enormously when X-rays act as well.

We irradiated (200 r) buds previously treated with EDTA; all buds in an inflorescence were treated with 0·001 M EDTA, starting 42 hr before the irradiation. The control inflorescences were in part treated with tap water; the treatment was stopped 4–5 hr before the irradiation. The two controls showed no statistically significant difference, though there was an increase in the crossovers if the water treatment was stopped half an hour before the irradiation because the buds were still wet. Irradiations at middle prophase damaged all chromosomes; some cells were arrested at prophase. The metaphase chromosomes stained very dark in the matrix; they behaved as if sticky. The crossovers were too numerous to be recorded accurately (Fig. 3, 4).

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The crossovers were readily recorded in cells irradiated in late interphase; the residual matrix was so slight that the spiral structures were readily seen (Fig. 3, 5, 6 and 10). The matrix was normal in cells irradiated at early interphase; the staining was only slightly less strong than in the controls (Fig. 3, 8, 9).

Table 2 shows that EDTA applied before irradiations which precede metaphase by 62 and 120 hr caused many more crossovers, as compared with cells not treated with EDTA. It may be that the EDTA broke some ionic bonds, but weakened far more; this could have resulted in more primary breaks in the chromosomes during irradiation, because the bonds were weaker. Figure 2 shows that the ratio of fragments to dicentrics plus rings was less when EDTA was combined with irradiation; this indicates that the number of recombinations was increased. The main feature is that EDTA and X-rays combined produced a type of crossover in interphase cells different from that produced by X-rays alone. Chromatid and semi-chromatid crossovers were also found (Fig. 3, 6, 8 and 9, Fig. 3, 10). Chromatid changes are produced by X-rays alone in prophase only; Swanson [17] first detected semichromatid crossovers on irradiating *Tradescantia* pollen tubes. These effects have subsequently been observed to occur spontaneously, and when chemical agents are combined with irradiation [18–21]; Sax states that they occur in *Tradescantia* microspores some 4–6 hr before metaphase, but at low (10–30 r) doses only. We found these crossovers produced at interphase by 200 r doses.

The ratio of dicentrics to rings is of interest. The dicentrics were produced when two chromosomes combined, the rings when two arms of one chromosome joined up. Irradiation at interphase produced a lower ratio of dicentrics to rings than did irradiation at prophase, probably because the chromosomes are less coiled up at interphase and are therefore much longer than in prophase, and because the chromosomes move more in prophase, particularly after breakage, so that different parts more readily collide. The ratio was higher if in addition EDTA was used as well at interphase,

which also indirectly shows that the chromosomes broken at interphase could join up again at later stages. The EDTA extended the time for which such joins were possible.

SUMMARY

(1) EDTA caused chromosome crossovers when used at a suitable concentration.

(2) The changes produced by EDTA and X-rays acting together were not additive.

(3) Chromatid and semichromatid crossovers occurred when the two acted together at interphase; the nature and the number of crossovers altered. This indicates that the EDTA increased the time during which broken chromosomes could join up. The new types of crossover that resulted from the joint action must mean that the types of mutations produced must also have altered.

I am indebted to Professor Gustafsson for his gift of *Tradescantia paludosa* plants of the Sax clone.

Translated by J. E. S. BRADLEY

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REGULARITIES IN THE DISTRIBUTION AND TOXICITY OF RADIOELEMENTS*

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THE effects of ionizing radiations on living matter and the use of the laws by which the interactions are governed for treating or preventing radiation injuries, or for discovering the mechanisms involved, are some of the most important topics in radio-

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biology. Discussion of the distributions and effects of radioisotopes constitutes only a small part of the radiobiological literature; very few data exist on the relations between the physical parameters (energy and type of emission, half-life), the physiological ones (distribution, elimination rate, etc.), and the type, length and outcome of any pathological process.

Distribution data enable us to compare the types and sites of damage in relation to the behaviour of the isotopes (etiology) and to calculate the maximum permissible body burdens of the emitters in man. Toxicological studies (i.e. the tissue doses required to produce some form of damage, such as cirrhosis, leukaemia, osteosarcoma or acute radiation death) can be based only on distribution data.

The data [1, 3, 5–7, 9, 11, 14–17] indicate that some elements (Sr, Ba, Ra, Y, Zr, Pu) are osteophilic, while others (La, Ce, Pm, Pr, Am, Cm) are selectively deposited in the liver; some accumulate largely in muscle (K, Cs, Rb), whereas others tend to prefer the reticulo-endothelial system (Nb, Ru, Te, Po). The rates of elimination from the bloodstream fall in the sequence Cs > Ba, Ra, Sr > Ce, La > Nb, Ru, Pu. The last three, with Te and Po, tend to circulate for a long time. Tri- and quadrivalent cations are absorbed and eliminated more slowly by the skeleton, kidneys, etc., than are uni- and di-valent ones. In some cases (Rb, Cs) the element migrates to the muscles, liver, etc., from the blood against the concentration gradient.

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The various groups in the periodic systems are fairly homogeneous in their behaviour. The elements of Group I (Li to Cs) are absorbed completely from the small intestine, are fairly evenly distributed, and are quite rapidly excreted in the urine. Those of Group II (Ca to Ra) are readily absorbed from the small intestine and deposit selectively in the skeleton. Some elements of Groups III and IV, including the lanthanides [7] and actinides, are hardly absorbed at all from the small intestine, and deposit selectively in the liver and (to a less extent) in the skeleton. Those of Groups V and VI, except Po, are readily absorbed and almost completely (70–80 per cent) eliminated in a few days in the urine; only small amounts remain in the organs.

The distributions cannot be represented schematically in any simple fashion; very similar elements sometimes show marked differences. The biological effects of the elements within a group may be very different; one cannot replace another without harm (beryllium and strontium poisoning, alkali disease); molecular processes are very important in pathology [1]. Four types of distribution can be distinguished (Table 1), namely, the skeletal, liver, uniform and kidney types. The type is clearly related to the main physicochemical properties; metals which give mostly water-soluble compounds give either the uniform pattern (Li to Cs, valency one) or the skeletal (Be to Ra, valency two). Elements which give basic hydroxides practically insoluble in water, and which are of valencies three or four, are mainly hydrolyzed to give radiocolloids in the body, with certain exceptions (Y, the heavy lanthanides); these give the liver type. Elements of valencies five to seven, which have acidic or amphoteric hydroxides, give either the uniform pattern (F, Cl, Br, Nb, Te, Po) or the liver one (Sb, As, S, Se, U^{6+}). In the last group the distribution depends very much on the state used. The elements (e.g. Be, Y, Ce) come out of true solution to give colloids when carrier is added or the pH raised; the distribution shifts from skeletal to liver. The skeleton and kidneys take up less, the liver and spleen more. Trace

amounts of Y (60 per cent) deposit in the skeleton, weighable amounts in the liver (up to 70 per cent). The uptake in the liver rises from 9·8 to 59·5 per cent on going from pH 1 to pH 13; the spleen values are 0·48 and 1·41 per cent, the skeletal 13·5 and 3·1 per cent, and the kidney 8·1 and 2·1 per cent.

TABLE 1. RELATIONSHIP OF DISTRIBUTION TYPE TO MAIN PHYSICOCHEMICAL PROPERTIES AND FORM IN WHICH THE ELEMENT IS ADMINISTERED

State	Typical hydroxide	Distribution type			
		Uniform	Skeletal	Liver	Kidney
Simple salts, trace amounts, pH 1–3:					
Valency 1	EOH	Li, Na, K Pb, Cs		—	—
Valency 2	$E(OH)_2$	—	Be, Ca, Sr	—	—
	HEO_2		Ba, Ra		
Valency 3	$E(OH)_3$	Ru	Y	La, Ce, Pr, Pm	—
	HEO_2			Ac, Pu, Hf, Th	
Valency 4	$E(OH)_4$	—	Zr	Pu, U, Am, Cm, Ce	—
Valency 5	H_3EO_4	Nb	P	—	Sb, As
	HEO_3				
Valency 6	H_2EO_4	Te, Po	Pu	—	U, S, Se
Valency 7	HEO_4	Cl, F, Br, I	—	—	—
Simple salts, trace amounts, pH 7	—	—	Sr	Be, Y, Ce, Ru	—
Simple salts, weighable amounts	—	Cs, Rb, K, Na, Ru	Ca, Sr, Ba, Ra	Be, Y, La, Ce, Pm, Am, Ac, Pu, Ca, Sr, Be, Y, Zr	Sb —
Colloidal solutions	—	—	—	Hf, P, Cr	—
Non-isotopic carrier	—	—	Sr, Ca	Y, Ce	Y, Ce, La, Pu
Chelates, etc.	—	Ru, Nb	Sr, Ba	—	Sb, U, Pb, P, Zr

Chelating agents (EDTA, hexametaphosphate) may alter the pattern; the skeletal or liver patterns of Ce, La, Y and Pu become kidney ones [3, 12]. No effects from pH, etc., are found with elements such as Rb, Cs, Ca, Ba and Sr.

Permeability changes are usually related to damage to vascular walls or to changes in the internal environment (the blood); the structures and properties of the tissues are not normally considered. Distribution experiments show that elements tend to have affinities for certain structures; the alkalis for the muscles (more so than other elements of the uniform type); the alkaline earths for the skeleton; and Ce, La, Y and Pu (with EDTA) for the kidneys. The capillaries must therefore be "selectively" permeable in a way closely related to the structure of the underlying tissue.

The absence of selective adsorption does not of course indicate that the capillaries are impermeable; Sr, for instance, is hardly deposited at all in muscle subcutaneous tissue, but bone tissue implanted in the skin takes up just as much Sr as normal (in the rat). The capillaries under the skin must therefore be quite permeable to Sr; the tissues are unable to bind it. Capillary permeability and tissue structure are thus closely related.

It is of course wrong to express doses only in terms of curies since the energies and masses of the particles, and the decay and elimination rates, are so different. The doses received should be put in radiation terms. Table 2 shows that the tissue doses

in radiation terms are more important than the activities required. In terms of μc/g ^{210}Po α-particles are 30,000 times more toxic than tritium [12]; the $LD_{50/30}$ values are respectively 0·03 and 1000 μc/g, but the effective tissue doses are roughly equal. The same applies to many other emitters; the $LD_{50/30}$ values for ^{239}Pu and ^{147}Pm are 0·05 μc/g and 7·0 μc/g respectively, for instance, while the effective doses given to the skeleton are about 4·6 and 2·8 krep respectively.

The distribution pattern is not very important in acute radiation death; the doses required from most β-emitters are about the same (2·2–5·4 krep).

The dose to the liver from ^{144}Ce (69·7 krep) is much larger than that from ^{90}Sr (0·1 krep); the mean tissue doses are about the same (5·3 and 4·1 krep, respectively), so it appears that the radiation to the liver does not accentuate the damage arising from irradiating the skeleton. The data do not confirm Friedell and Christie's [10] results (that liver irradiation acts additively with bone-marrow irradiation); they show that the direct bone-marrow damage is the major factor in acute radiation sickness.

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TABLE 2. TISSUE DOSES (IN KREP/g) PRODUCED IN ORGANS 30 DAYS AFTER INJECTING $LD_{50/30}$ AMOUNTS OF EMITTERS

Isotope	$T_{1/2}$	Quantity for $LD_{50/30}$ (μc/g)	Skeleton	Liver	Kidney	Spleen	Bone marrow	Other organs	Mean* dose
Cs-137	33 years	21·0	2·7	3·4	4·8	2·7	2·7	6·5‡	4·5
Sr-89	53 days	4·5	54·0	—	—	—	—	—	5·4
Sr-90	20 years	1·5	40·5	0·1	—	—	—	—	4·1
Ba-140	12·8 days	2·0	24·0	2·5	—	—	—	—	2·8
Ra-226	1590 years	1·5	135·0†	—	—	—	—	—	13·5
Y-90	61 hr	7·0	16·1	0·9	4·4	—	—	—	2·3
Y-91	61 days	3·7	18·3	1·2	3·6	—	—	—	2·2
Ce-144	282 days	3·4	27·2	69·7	10·6	—	—	—	5·3
La-140	40 hr	14·5	3·9	22·0	—	—	—	—	1·6
Pm-147	2·6 years	7·0	2·8	4·2	—	—	—	—	0·7
Zr-95	65 days	7·4	15·6	2·5	—	—	—	2·1	3·1
Nb-95	35 days	8·4	2·0	1·6	2·4	2·7	1·5	—	1·1
Ru-106	1 year	3·7	5·5	8·6	29·0	19·0	4·7	—	3·2
Pu-239	$2{\cdot}41\times10^4$years	0·05	4·6	0·5	0·25	—	—	—	0·7
Po-210	138 days	0·03	—	0·4	1·6	3·6	0·9	—	—
U-233	$1{\cdot}63\times10^5$years	0·04	1·3	—	1·8	—	—	—	—
T	12·4 years	1000	1·0	—	—	—	—	1·0	1·0

* Mean doses calculated assuming uniform distributions.

† The tissue doses for Ra have been calculated assuming all daughter elements eliminated; they are therefore too low. Assuming 25 per cent of the daughter products retained in the skeleton, the value would be 265 instead of 135.

‡ Muscles.

Table 2 gives rise to some other important conclusions. It is commonly assumed [4] that α-emitters are by four to twenty times biologically more effective than β-emitters, though the problem is too complex for this to be assumed in any general form. The effectiveness of an α-emitter depends on precisely how it is distributed in the tissue; an α-emitter may be less effective than a β-emitter if much of the energy is absorbed in insensitive materials or cells (e.g. calcified cell).

^{226}Ra is less effective than the similar β-emitters ^{140}Ba, ^{90}Sr, ^{90}Y and ^{89}Sr. Much higher doses (135 krep) are required to produce acute death with Ra in the skeleton than are required with these β-emitters (18–54 krep). The soft β-emitters resemble α-emitters (^{95}Nb, T, ^{147}Pm and ^{210}Po, ^{239}Pu produce about equal integral doses—see Table 2), but enormously higher β-activities are required (factors of 150 to 280 are involved; equivalent doses are given by 0·03 μc/g of ^{210}Po, 8·4 μc/g of ^{95}Nb, 0·05 μc/g of ^{239}Pu and 7·0 μc/g of ^{147}Pm). The elements are distributed in roughly the same way in the tissues, so the α- and soft β-particles do not differ in biological effectiveness.

The effectiveness of β-particles depends on the energy; the soft emitters (^{95}Nb, ^{147}Pm, T) are usually more effective than the hard ones (^{144}Ce, ^{106}Ru) at the acute level, because the ends of the tracks are particularly effective; the number of ends is proportional to the number of decays, and not to the dose. The $LD_{50/30}$ for T is 1000 μc/g, while for ^{106}Ru it is 3·7 μc/g; the mean tissue doses are 1·0 and 3·2 krep respectively.

Single doses of X- or γ-rays (from ^{60}Co) are more effective than those from β-emitters in the body; the $LD_{50/30}$ value for the first is 400–600 r for rats, while isotopes require 1·1 to 5·4 krep. The β-emitters are less effective because the dose rates are low and the emitters are unevenly distributed. This agrees with the fact that X- and γ-rays are less effective at the absolute lethal dose level if the doses are fractionated [2, 13]. High dose rates are also more effective with radioisotopes; ^{144}Ce (half-life 282 days) and ^{140}La (half-life 40 hr), ^{90}Sr (half-life 20 years) and ^{140}Ba (half-life 12·8 days) show identical distributions. The short half-lives of ^{140}Ba and ^{140}La mean that the dose rates are much higher at the start; the doses are 1·6 krep and 2·8 krep, as against 5·3 krep for ^{144}Ce and 4·1 krep for ^{90}Sr. This shows why most deaths occur in the first two weeks with the short-lived materials.

Further data are required on the distributions and biological effects of radioisotopes, as these data are exceptionally important in radiobiology, in relation to the connections between the distribution type, physical properties of the emitter, and the type, duration and extent of the damage.

SUMMARY

(1) Some regularities in the distributions and biological effects of radioisotopes are discussed.

(2) The elements in any one group of the periodic system behave very similarly. The types of distribution distinguished are the skeletal, the liver, the uniform and the kidney.

(3) Complexing agents, carriers and pH alter the distribution types found with elements having hydrolyzable compounds; they produce no effects with elements giving mostly soluble compounds.

(4) Toxicities should not be evaluated in terms of activity alone, since the radiation dose is the primary parameter, and not the decay rate. The effectiveness of any emitter depends on its physical parameters (energy, emission type, half-life) and on its physiological behaviour (distribution type, rate of elimination, degree of absorption from depots).

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METHODS AND APPARATUS

MANUFACTURE OF GLASS KNIVES FOR ULTRAMICROTOMES*

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The glass knives first used in ultramicrotomes by Latta and Hartman [1] are now in common use, although techniques for use with steel knives are highly developed [2–4]. Glass knives are much easier to make, and are cheaper, than steel knives. However, some practical rules have to be followed if good glass knives are required.

(1) *Choice of glass.* It has been stated that the best glasses are those of high silica content [5]. We have tested some glasses presented to us by the All-Union Glass Research Institute; these were all high in silica and variously annealed. They were unbleached glasses (Estonian, Gor'kov, "proletarian", white), a borosilicate and a Konstantinov plate glass. The glass used in Sjöstrand's ultramicrotome, presented

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TABLE 1. COMPOSITIONS OF THE GLASSES

Type	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	NiO_2	SiO_3	Na_2O	K_2O	Ba_2O_3
	%%									
Unbleached:										
(*a*) Gor'kov	72·0	0·45	0·09	8·28	3·58	14·82	0·42	14·82	—	—
(*b*) Estonian	72·0	0·45	0·09	8·28	3·58	14·82	0·42	14·82	—	—
(*c*) "proletarian"	72·0	0·45	0·09	8·28	3·58	14·82	0·42	14·82	—	—
(*d*) white	72·0	0·45	0·09	8·28	3·58	14·82	0·42	14·82	—	—
Borosilicate	71·4	0·2	—	1·4	0·4	—	—	7·6	11·2	7·8
Konstantinov plate glass	72·4	0·5	0·08	12·4	0·1	—	0·42	14·1	—	—
Swedish glass*	72·0	TiO_2–1·04	0·08	8·84	3·45	—	0·42	13·05	0·88	—

* This glass is used in Sjöstrand's ultramicrotome, produced by LKB.

by the LKB Company, and the types of glass used in Sjöstrand's laboratory, were also tested. Table 1 gives the data on the compositions of the glasses.

(2) *Preparation methods.* The usual methods have been adequately described [1, 3, 6]. We used a somewhat unusual method. The glass was first degreased (with warm water and soap), cut into strips 25–30 mm wide and marked with a glass

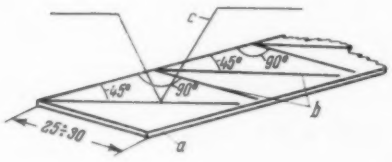

FIG. 1. (*a*) Glass plate. (*b*) Markings. (*c*) Nichrome wire.

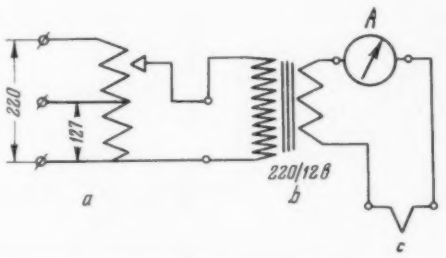

FIG. 2. (*a*) Autotransformer (LATR-1). (*b*) Reducing transformer. (*c*) Nichrome wire.

knife at 90° and 45°, as shown in Fig. 1. The strips were cooled to between −5° and −7°C. The strips were not cracked in the usual way [6], or with an incandescent pyrex rod [7], but with a nichrome wire heated to 900–1000°C. The device used (Fig. 2) consisted of a LATR-1 laboratory transformer, a 220/12 (50 A) step-down transformer, a meter to read 50 A, and a holder for the nichrome wire (1–1·5 mm). Currents of 20–28 A were used, with 7–8 cm lengths of wire bent into a V. The best side of the plate was used (the one with the least scratches).

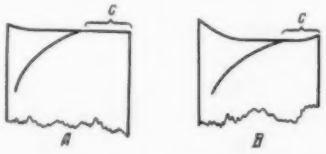

FIG. 3. (*C*) Region usable beneath the cutting edge.

(3) *Checking for suitability.* The sections cut give the best guide to the quality of a knife, but it was possible to assess the quality from the form of the cutting edge; those with flat surfaces (at C) were the best (as in Fig. 3*a*). Those knives which showed a fine bright line under dark field illumination were the best, while those

TABLE 2. YIELDS OF USABLE KNIVES

Glass type*	Yield of knives† (%) on annealing of varying extent (mm/cm)					
	5	19	21	25	40	57
Unbleached:						
(*a*) Gor'kov	20	45	51	50	26	15
(*b*) Estonian	18	41	48	45	21	10
(*c*) "proletarian"	13	31	30	24	17	12
(*d*) white	5	10	25	—	15	8
Borosilicate	10	17	—	23	—	10
Konstantinov plate glass	14	19	—	17	—	10
Swedish glass‡	—	—	39	—	—	—

* The strips were 5 mm wide.
† Of high grade.
‡ This glass is used in Sjöstrand's ultramicrotome, produced by LKB; width 9·5 mm/cm and annealing 21 mμ/cm.

that showed broken, jagged or diffuse lines were poor [8]. Our checks were done very simply; the jaws of a normal slide holder fastened to a microscope stage (Zeiss Jena) were made to hold the piece of glass vertically in the hole in the stage. The cutting edge was seen bright against a dark background under oblique illumination (from an OI-7 illuminator). A low magnification was used at first (objective ×40, eyepiece ×15). Suitable regions for cutting were usually places where a ripple emerged (as in Figs. 3*a* and 3*b*); these regions varied in width from 0·05 to 1 mm. The best results were obtained with knives set at 3–5° to the vertical.

RESULTS

The unbleached glasses gave the best results, not inferior to Swedish glass. A temperature of −5 to −7°C is best; the results are not so good at higher or lower temperatures (the numbers of good knives are less, and the cutting edges worse, with jagged edges and reduced widths of good area). The degree of annealing is important; we find the 18–30 mμ/cm range best. Table 2 gives the results; the best width was found to be about 5 mm.

SUMMARY

(1) The making of glass ultramicrotome knives from Soviet glasses is considered.

(2) Unbleached Estonian and Gor'kov glasses, annealed to 15–25 mμ/cm, and 5 mm wide, are best.

(3) A simple device of assistance in making the knives is described.

(4) Preliminary cooling to between −5 and −7°C improves the yield of good knives.

The authors are indebted to O. V. Krasnovskii and A. I. Kolbasinkova for specimens of the glasses, and for undertaking the analyses.

Translated by J. E. S. BRADLEY

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LETTERS TO THE EDITORS

THE ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF NATIVE AND DENATURED PROTEINS*

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(*Received* 20 *June* 1958)

THE electron paramagnetic resonance (e.p.r.) spectra of irradiated biological objects have been recorded by many workers; Gordy and his group [1] have made the most extensive studies. Our independent results of 1956–1957 in some instances agree with those of Gordy, and in some disagree. Agreement is found for irradiated amino acids, which show high yields of free radicals and strong broad e.p.r. signals, mostly with hyperfine structures susceptible of an acceptable structural interpretation. Disagreement exists over proteins and freeze-dried tissues [1]; the differences are fundamental, and form the subject of the present note.

Most of our irradiated native proteins and freeze-dried tissues gave yields much lower than did the amino acids (factors of 10^3–10^4 are involved), while the lines themselves are narrow (2–4 oersted) and structureless. Proteins and tissues, thermally denatured before irradiation, in all cases gave broad strong signals of the 16 oersted doublet splitting typical of isolated hydrogen bonds. These doublet signals have been detected in many irradiated native proteins by Gordy's group, but they have done no tests on prior denaturation; in most cases they have used 50–60 kV X-rays and doses of about 1 Mr. Soft X-rays tend to heat the specimens somewhat; the photoelectric effect which causes the heating is responsible for some 50 per cent of the absorption at 50–60 kV.

We have always used ^{60}Co γ-rays, which give only a very small photoelectric yield. Some tests with soft X-rays, and very high dose rates from ^{60}Co, show that the protein is thermally denatured in the beam (the temperature rises to 70–80°C). We therefore explain the discrepancies as due to denaturation during irradiation in the American work.

Translated by J. E. S. BRADLEY

* Biofizika **3:** No. 6, 735, 1958.

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* There exists a translation into English of this work.

THE RELATION BETWEEN THERMAL AND ULTRA-VIOLET DENATURATION IN PROTEINS*

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PROTEIN molecules are stabilized largely by their intramolecular hydrogen and salt bonds. All the bonds are affected simultaneously when a protein is denatured, thermally or otherwise; the number of weak bonds broken, n, may [1–3] be found approximately from the ratio of the heat of activation for denaturation ΔH^* to the mean intermolecular bond energy, e; it is approximately 10 for pepsin, albumin, haemoglobin, etc. In ultra-violet denaturation the bonds are broken one at a time; some bonds may join up again in the old way. Each bond is broken independently, so the probability of breaking n bonds simultaneously is the probability p of breaking one bond raised to the power n. The above value of n implies that the quantum yield Φ must be very small, as is in fact found.

Now Φ is proportional to p^n, or

$$\Phi = \Phi_0 p^n \tag{1}$$

where $\Phi = \Phi_0$ when $n = 0$. The data on thermal denaturation [1–3] give

$$n = \Delta H^*/e. \tag{2}$$

Substituting (2) into (1) and taking logarithms, we have

$$\log \Phi = \log \Phi_0 + (\Delta H^* \log p)/e \log \Phi_0 + (\log p\, \Delta H^*)/e. \tag{3}$$

Putting $k = (\log p)/e$, we have

$$\log \Phi = \log \Phi_0 + k\Delta H^*. \tag{4}$$

ΔH^* and $\log \Phi$ are thus linearly related. The data collected in Table 1 can be used to check this relation, although they give only a rough check of (1). Although Φ and ΔH^* have not been determined simultaneously for identical proteins under comparable conditions, values of both are in any case available for only a few proteins. As measurements at identical concentrations, buffer compositions, pH, etc., would be most laborious, we can but compare the various published data.

The figures show that (4) is verified reasonably accurately; when $\Delta H^* = 0$ (i.e. $n = 0$) $\log \Phi_0 = -1$, so $\Phi_0 = 0{\cdot}1$; this is an upper limit to the quantum yield,

† Biofizika **3**: No. 6, 736–737, 1958.

TABLE 1. VALUES OF ΔH^* AND Φ FOR DENATURATION

Protein	M.W.	ΔH^* (kcal/mole)	Φ	log Φ
Insulin	12,000	35·6 [4]	$1{\cdot}5\times10^{-2}$ [8]	−1·82
Lysozyme	14,000	30·0 [5]	$2{\cdot}4\times10^{-2}$ [9]	−1·62
Trypsin	23,000	40·1 [4]	$1{\cdot}8$–$2{\cdot}1\times10^{-2}$ [10, 11]	−1·76
Trypsin inhibitor	24,000	55·0 [6]	$6{\cdot}10^{-3}$ [12]	
			$8{\cdot}8\times10^{-3}$ [8]	−2·15
Pepsin	33,000	55·6 [4]	$2{\cdot}1\times10^{-3}$ [13]	−2·68
Catalase	250,000	87·0 [7]	$1{\cdot}0\times10^{-3}$ [11]	−3·0
TMV	42,000,000	153·0 [3]	$4{\cdot}3\times10^{-5}$ [10, 14]	−4·37

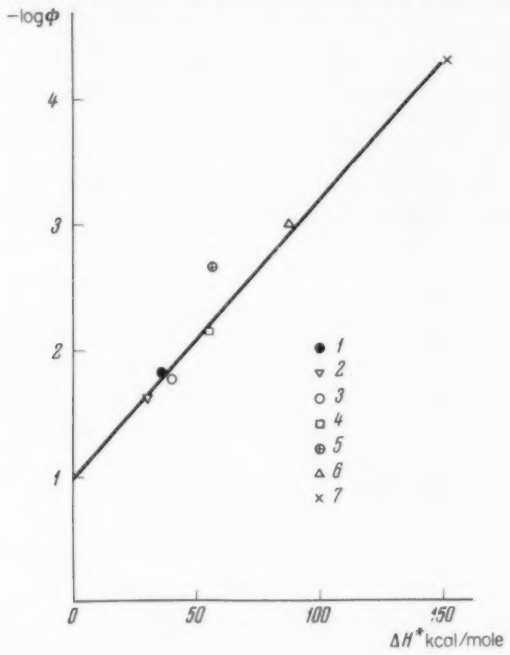

FIG. 1. log Φ vs. ΔH^* for proteins. (1) insulin, (2) lysozyme, (3) trypsin, (4) trypsin inhibitor, (5) pepsin, (6) catalase, (7) tobacco mosaic virus.

and all known values are such that $\Phi < 0{\cdot}1$. We also find that $k = -0{\cdot}0214$, so (4) becomes

$$-\log \Phi = 1 + 0{\cdot}0214\Delta H^*. \tag{4a}$$

This relationship covers the entire molecular weight range shown by proteins (12×10^3 to 42×10^6); so Φ can be calculated from ΔH^* and vice versa.

Now $k = (\log p)/e = -0{\cdot}0214$ is constant, so the mean energies of the weak intramolecular bonds and the mean rupture probabilities are related. If $e = 1{\cdot}5$–$2{\cdot}0$ kcal/mole [15] we have $p \sim 0{\cdot}90$, and if $e = 5$ kcal/mole [2] we have $p \sim 0{\cdot}78$. The broken bonds must be able to join up again as $p < 1$.

More bonds must be broken in the larger molecules, so (1) implies that the quantum yield must fall as n increases since $p<1$; this has been found empirically by McLaren [14, 16].

The author is indebted to A. M. Tongur for assistance in assembling the published data.

Translated by J. E. S. BRADLEY

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DISCUSSION

THE STATE AND ROLE OF WATER IN BIOLOGICAL SYSTEMS*

P. L. PRIVALOV

(*Received* 12 *May* 1958)

THE state and role of water in biological systems are often said to be special, not merely as a result of purely speculative explanations of imperfectly understood phenomena in terms of a special state of the water, but also because direct physical studies of water have advanced greatly in the recent past [1].

The structure of water is now held to be determined largely by the hydrogen bonds which join each molecule to four of its neighbours; the structure is very open, with holes larger than the molecules themselves (Fig. 1). Water differs from ice mainly in that the long-range order is lost. The hydrogen bonds bend and break during thermal oscillations [2, 3]; molecules which have left their equilibrium positions fall into adjacent holes and remain there for a certain time, since the holes represent relative minima in potential energy. The co-ordination number is increased

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FIG. 1. Structure of ice.

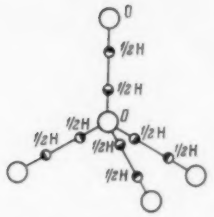

FIG. 2. The proton distribution round the oxygen atoms in water.

thereby, and lattice defects are formed; these defects are responsible for the anomalous properties of water [1].

The hydrogen bonds join any given mass of water into a united system; and some authors tend to treat this system as if it were one giant molecule [4]. Neutron diffraction has shown [5] that the number of states open to the protons is twice as large as the actual number of protons; every oxygen atom is joined to two protons which can occupy four positions quite indifferently. Figure 2 shows that half a proton occupies each possible position. Proton transitions from one position to another can be considered either as molecular reorientations or as the formation of ion pairs; the activation energies of the two processes for ice are 12·4 and 8 kcal/mole respectively [6]. The proton transitions cannot be entirely independent, since charge shifts and hydrogen bond changes are involved; molecular reorientations are therefore correlated, and ordered molecular groups and chains are formed. The less the activation energy the more frequent the transitions, and hence the shorter the chains. The activation energy in water is only 2 kcal/mole, so there are few orientated chains. Ordering can thus be considered as equivalent to fixing the proton positions; the cause of the anomalous proton mobility of water is thus clear. Grottus' mechanism implies that the form of the free state of the proton is displaced, and not an actual proton, i.e. the proton joins on to the nearest water molecule and displaces another proton, which repeats the process; the effect is one of proton migration.

Ril' has made a very detailed study of proton mobility in water [6], and has measured the electrical conductivity of ice. The probabilities of the possible positions are altered by placing the ice in a strong electrostatic field; the dipoles show a preferred orientation and chains are formed. It is more probable that an orientated molecule will be produced in front of or behind a molecule already oriented, because the dipole field is stronger than the field at other points. The proton can "propagate" extremely rapidly along such an oriented chain, without encountering any significant potential barriers. Figure 3 shows that the chain is incorrectly oriented after the proton has passed along it, so the process cannot repeat; the probability of the chain's breaking

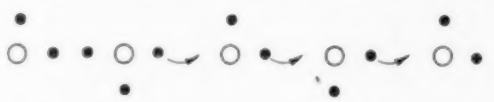

FIG. 3. The Grottus proton displacement mechanism.

is also thereby increased. The chain breaks up, and then reforms correctly. The mechanism can be considered as one of proton breakdown along the oriented chains.

The charges on the dipoles affect the losses in alternating fields as well as the d.c. conductivity. The proton mobility within a chain is very high and very much larger than for the ice as a whole. A conductivity will therefore be found at high frequencies so long as a proton has time to pass from one end of a chain to the other during a half-cycle; Murphy [7] has detected this conductivity. Water does not show the effect, because the chains are short and of low concentration.

The ordered structures in water thus result in energy migration effects (transmission of energy to a distance without loss). Any disturbance to the proton at one end of a chain must cause chain reorientation; if a free proton joins on to one end, another free proton is produced some distance away, at the expense of disordering the orientated structure. If dipole ordering somehow appears in a disordered system of water molecules (e.g. the protons are fixed in definite positions, say about some surface) the ordered state must be propagated and must result in an oriented layer of water, because the proton positions are correlated.

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Ril' has shown that the electrical conductivity of a strong solution of gelatin at high fields is such that the activation energy is similar to that for ice. The only possible explanation is that the protein orients the water molecules and gives the water properties similar to those of ice. Proton transfer along chains is rapid in such circumstances. The transfer disturbs the orientation (the ice, as it were, melts). Ril' considers that his data on the conductivities of organic compounds [9, 10] show that proton transfer is quite possible in proteins; the channels of least dielectric strength break down. This is one of the possible energy migration mechanisms for proteins.

Ril's experiments are not the only proof of the ordering effects of macromolecules; proton resonance and high-frequency dielectric measurements are very important. The line width of the proton resonance absorption depends on the spin-lattice interaction [11, 12], and hence on the state of the material; the lines broaden greatly when a liquid crystallizes [13]. The state of the water in biological objects can therefore be studied via proton resonance line-widths. The line width increases considerably when macromolecular compounds are dissolved [14–17]; the lines from tissues are also broad. The water must therefore be ordered.

Dielectric measurements give the most accurate results, although the high losses in biological objects restrict the use of the method. Water and ice have very different dispersion regions because their proton mobilities differ [18]. The composition of an ice–water mixture can be determined by making dielectric measurements in the interval between the dispersion regions of the two phases (Fig. 4) [19]; the method has also been used with moist clays [20, 21] and solutions of macromolecules [22–24]. Surfaces can order water layers several microns thick [19].

The dielectric behaviour of aqueous DNA (flow-orientated) is of particular interest [22]; the DNA molecules can order the water out to 100 mμ from their surfaces, i.e. to a depth equal to one quarter of the length of the molecule. The interface with the water is very important. Asymmetric molecules are highly effective in ordering the water; the capacity to order depends very much on the structure of the surface (number of groups which form hydrogen bonds). The closer the structure

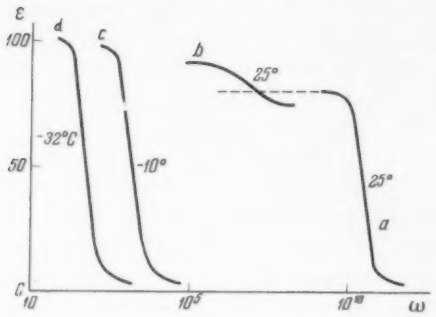

FIG. 4. The dielectric constants of water (+25°C, *a*), of ice (−10°C, *c*; −32°C, *d*), and of a solution of a macromolecular compound in water, *b*.

to that of ice the greater the capacity to order. The molecules in frozen solutions approach the ice structure more closely, and their ordering power increases [23]. The high effectiveness of DNA occurs because DNA has a spiral structure which fits the lattice of ice perfectly [24]. Electrolytes cause the spiral to contract; it does not fit the ice so well, and the water is much less ordered [17, 24]—a very important point.

The ordering of the water is not such as is found in hydrated ions; hydrated ions do not have the normal structure of water, because the field of the ion breaks up the water structure. The hydrogen bonds of macromolecules merely stabilize the water and "reduce the structural temperature" [22]. This action is dependent on groups which form hydrogen bonds, which are also responsible for the solubility in water; a substance which gives very few bonds to the water is insoluble. It has, however, been shown that even sparingly soluble compounds which form no hydrogen bonds can order the water. The explanation is that such molecules leave the internal pressure of the water uncompensated; the freezing point of water is raised by decreasing the pressure, so the boundary layer "freezes" and forms what have been called "icebergs". Some authors [25] attach special importance to this effect in biological systems, since protein molecules have many inactive groups.

Most, if not all, of the water in a biological system which is in contact with surfaces will be ordered. We can for simplicity imagine that such a system is a completely ordered structure with periodic fields, but this must imply that proton transfer can occur. Any disturbance propagates as a wave of disorder, which is damped because a certain dissociation energy is required. The wave cannot be maintained unless an energy source exists to maintain it; the source can be a metastable state of structural type. The disturbance causes a transition to the stable state, which liberates energy and causes the disturbance to spread. Various types of metastable state are possible; the most interesting relates the state directly to the structure of water [26]. The chemical energy is stored as energy of electron triplet-excitation. The triplet state can persist only if the electromagnetic field in the system has a regular character; any disturbance to the field causes the triplet state to go over to the stable ground state. The energy so liberated causes the process to continue. The feedback loop in the chain reaction of disturbance to energy released from the metastable state to

still greater disturbance is thus closed. Any disturbance can trigger off this reaction, but the situation is also continuously being restored. Small disturbances are therefore incapable of starting the reaction, since the smaller the disturbance the more rapidly it is eliminated and the less its effect on the metastable state. The amplification factor K is less than one for small disturbances; K increases with the scale of the disturbance, and when $K > 1$ the reaction diverges; the system is completely disrupted. The rate of spread depends on how fast the various stages follow one another.

Hence fluctuation effects, although always present, will not cause the system to be disrupted; separate small disturbances are weakly correlated, and a certain threshold has to be exceeded before the interaction leads on to full disturbance.

The system rapidly begins to re-order itself after a disturbance; heat is liberated and the structure restored. The metastable levels are occupied again; the energy is derived from the chemical reactions in the system. The ions are redistributed between the system and its external medium during disturbance and restoration; the effects are dependent on how many ions can be accommodated in the ordered water. The effect has been studied in relation to the ordering that occurs on freezing water [27]; a large potential difference is produced at the interface between the phases, which may on occasion be of 0·01 V magnitude, i.e. quite adequate to account for the high voltages developed by certain animals. The ionic currents facilitate the spread of the disturbance wave.

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We may well suppose that all forms of excitation involve the same basic mechanism, which merely shows a shift in the precise balance between its various aspects in different cases. The main component of muscle which can order the water is actomyosin; this can alter in configuration by contracting. The initial configuration may be taken as metastable; after discharge the molecule may not resemble water in structure; the disturbance which results from the transition in one molecule may spread to others and trigger them off. Excitation processes in other systems may also start from configuration changes in macromolecules.

The ions may be considered as the main structure regulators, and as the main regulators of bioenergetic processes generally [26]. Their actions depend on the changes they cause in the ordered structure, how they fit into the water lattice, and how strongly they disturb the metastable state. The effects of ionizing radiations may be considered in this connection.

A biological system can be considered as a single ordered whole in which effects can be transmitted to a distance via the proton transfer mechanism; the direct and indirect effects of radiation are connected. The radicals produced by irradiation do not simply diffuse through the medium; they migrate by the Grottus' mechanism and are restricted only by such discontinuities as may occur. Damage will therefore build up at the surfaces of the macromolecules; the charge distributions will be altered and the hydrogen bonds broken. Damage will be concentrated by migration; some authors [28] have explained in this way why biological systems are so sensitive to ionizing radiations. The effects will clearly be the more marked the more highly ordered the system. This explains why the more highly organized an organism the more radio-sensitive it is [29].

We have of course supposed that all our assumptions about the structure, migration and metastable states are correct; this is not necessarily so, but the whole discussion need not be rejected out of hand. The results are of interest in that they

show how some bioenergetic processes can be analysed precisely, and what the possible role of the structure is.

I am indebted to L. P. Kaiushin, Ya. L. Shekhtman and L. A. Tumerman for reading and criticizing the manuscript.

SUMMARY

(1) Biological systems can be considered as unities with ordered structures, since it has been shown that the water is in an ordered state; effects can be transmitted to a distance by proton transfer. Any disturbance is transmitted as a wave of structural disorganization.

(2) If a certain structure corresponds to a metastable state, and any disturbance to the structure liberates energy, we have a feedback mechanism; this may explain the avalanche character of excitation processes.

(3) The action-at-a-distance mechanism must be important in relation to the effects of ionizing radiation on biological systems, since it enables damage to migrate and become concentrated on intracellular structures. It explains the exceptional sensitivity to irradiation, and to some extent provides a common mechanism for the direct and indirect effects.

Translated by J. E. S. BRADLEY

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* There exists a translation into English of this work.

POSSIBLE WAYS OF DETECTING ELECTRON CONDUCTION IN NERVOUS ELEMENTS*

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ERNST [1] has proposed an interesting semiconduction mechanism for excitation and transmission at synapses, which has not, however, been verified. The known facts are not explained and new effects are not predicted. The semiconductor concepts and systems considered are a very rough approximation; for example, excitation is not related to the Hall effect (there is no magnetic field) nor to Schottky's law; electrons and holes cannot be thought of as in definite states in semiconductors; the mean lifetimes of the carriers are not related to the durations of the processes in semiconductor circuits. The problem itself has not been formulated properly, since uni-directional conduction and conductivities varying with impurity content and temperature are found in ionic conductors as well. It has repeatedly been shown [2, 3], and cannot be denied, that ionic conduction is very important in nerves.

There is only one correct way to pose the problem, namely whether electron conduction plays any real part in nervous systems. An answer can be given from direct studies on the types of conduction shown by the various nervous elements. Simultaneous conductivity and Hall effect measurements give the most useful data. If a Hall e.m.f. is detected the carriers must be moving fairly rapidly; this can occur in electron conductors only.

Electron conduction in some parts of the nervous system was demonstrated long ago; and some workers [4, 5] have sought to detect it from the effects of fixed or slowly varying magnetic fields on nervous operations. A fixed field can act on moving charges and nuclear or electron moments only. No physicochemical effects are known which could cause any appreciable potential differences across membranes by acting on nuclear or electron moments alone. The magnetic field must act on fast-moving particles, i.e. electrons or holes.

It has also been shown that magnetic fields alternating at 10–90 c/s act on the human eye [6–8]; the light appears to flicker at the same frequency as the field. There is no effect in bright light or in darkness. The induced e.m.f. that the alternating field produced could not cause the effect because it was some thousands of times too small. The effect cannot arise from para-, dia- or ferro-magnetism, since the frequency would then be twice that of the field.

The flickering implies that the retinal nerve cells produce impulses, which [3] they do when their membranes are depolarized. The field could cause such an effect via the Hall or photomagnetic effects. A rough calculation shows that the Hall e.m.f. will be large enough if the carrier mobility exceeds 10^3 cm^2/V sec; only electron-type carriers can have such mobilities. The lack of an effect in darkness supports this

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conclusion; the membrane is carrying no current in darkness, so there can be no Hall effect. An electric field can cause the appearance of light even in darkness, though the sensitivity is reduced; it is not clear whether the reduction is sufficient to explain the lack of an effect if we assign the effect to Foucault currents.

Electron conduction, if present, would enable us to explain how photoreceptors and other nervous elements operate. The diagram shows a simplified semiconductor circuit design as an analogue to a myelinized nerve fibre. Germanium double-base diodes D [9] are used; their resistances can be controlled via the voltage on the emitter E. The battery B_1 provides the constant polarization; P_1 resembles a Ranvier node. At rest the resistance between points b_1 and b_2 on D is [8] large, since the potential on E is less than at the corresponding section of the base d. The stimulating current must [10] pass through the Ranvier node in such a way that positive charges move from inside to outside; a current in this direction reduces the potential difference between b_1 and b_2. The potential at d is below that at E, and is held there for a certain time by the capacitor C. A current flows from E to the base and increases the carrier concentration; the resistance between b_1 and b_2 drops sharply. The charged line L, which resembles the fibre between nodes, begins to discharge through the diode and (together with battery B_2) produces an action current of reverse sign to the stimulating current. The discharged line is then charged by the B_1 in the second node; the current which flows stimulates the second node, since it reduces the potential differences between the bases of the second diode. This diode begins to conduct, and the impulse is transmitted. A current directed back into the node will not cause the diode to conduct.

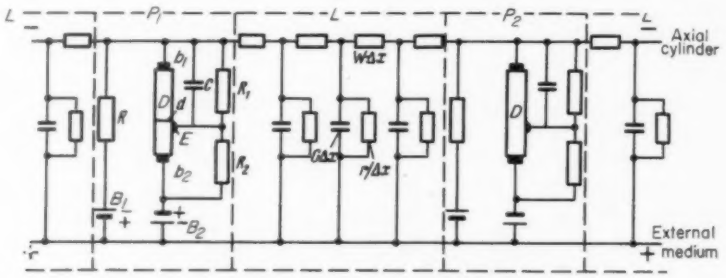

FIG. 1. Model of a nerve fibre.

The action currents from single fibres give oscillograms similar to those from the analogue. The model can also give the catanelectroton and other effects if slightly modified.

This model is not, of course, unique; others can be built round other semiconductor devices, or round valves. Semiconductors are convenient in that they can work at 100–200 mV. The semiconductor components in real nerves may be protein molecules, which can show electron conduction under certain conditions [11].

Direct tests for electron conduction in nerve elements would be of value. Photoconductivity, photo-e.m.f. and thermo-e.m.f. measurements should be made to supplement the magnetic ones.

Translated by J. E. S. BRADLEY

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CURRENT EVENTS

THE INTERNATIONAL RADIOBIOLOGICAL CONGRESS*

A. M. Kuzin, N. A. Bakh, M. N. Meisel', M. N. Pobedinskii and V. A. Petrov

The congress was held at the University of Vermont, Burlington, U.S.A., from 10 to 16 August 1958. There were about 800 persons at the congress, 650 from the U.S.A. and 150 from 25 other countries. Some 350 papers were read. There were two plenary sessions, together with ten sections and seven symposia that ran simultaneously. General papers that dealt with the various branches of radiation research were presented at the plenary sessions. The sections dealt with the following topics:

1. Biophysics and dosimetry.
2. Radiation chemistry.
3. Radiation biochemistry.
4. Cellular radiobiology.
5. Radiation physiology.
6. Radiation damage in vertebrates.
7. Protection and recovery.
8. Radiation genetics.
9. Effects of internal emitters.
10. Radiation and carcinogenesis.

The symposia dealt with:

1. Oxygen in radiation chemistry.
2. Free radicals.
3. Primary processes.
4. Radiation effects in yeasts.
5. DNA and chromosomes.

6 and 7. Remote after-effects of radiation in mammals.

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I. BIOPHYSICS AND DOSIMETRY

Eighty-nine of the 350 papers were read in this section; the papers on radiobiology and radiation damage dealt also with many biophysical topics. There were 28 papers on dosimetry as applied to biological media, nearly all of which were from the U.S.A.; this is partly explained by the congress being held in the U.S.A. (Table 1). All forms of radiation were dealt with, though neutron dosimetry, with only two papers, was inadequately represented (Table 2).

TABLE 1. NATIONAL ANALYSIS OF THE PAPERS PRESENTED

Country	No. of papers
Great Britain	2
Canada	1
U.S.S.R.	1
U.S.A.	24

TABLE 2. ANALYSIS OF THE PAPERS ACCORDING TO TYPE OF RADIATION

Radiation	No. of papers
α	4
β	7
γ	5
X	5
General	5
Neutron	2

Frigerio's paper on tissue-equivalent systems for neutron dosimetry was of great interest; the characteristics of formaldehyde, agar, alginate and acrilin were given. Absorbed-dose measurements (unit: the rad) were not considered, although the topic is a basic one in dosimetry today. Only one paper, by Gross and others (from the radiation laboratory at the University of Colombia), dealt with direct dose measurement in rads. Tissue- and air-equivalent media were used. This was a first step towards direct measurements of absorbed doses in rads.

New methods (including the Monte Carlo) have been used to calculate X- and γ-ray scattering. The Monte Carlo method is most promising, since very complex and laborious calculations can thereby be systematized. Many of the papers dealt with refinements to details or to numerical data that had been derived previously. Shumway and others gave sets of dose-distribution curves for tissues in the electron beam from a Van der Graff accelerator, for particle energies of from 0·5 to 2·0 MeV. The measurements were made very accurately, and so the results can be used as standard values.

Much work was presented on the spectra of emitters and of scattered radiations. These studies of spectra are themselves of interest, and are also used to solve physical and biophysical problems.

A notable feature was that physical methods are now being widely used in radiology and radiobiology; physicists proper are working in these fields. It is clear from the papers that research institutions dispose of radiation sources which are very varied in both type and strength. Very many types of physical instrument, including very complex ones, are used in measurements. The illustrations that were given with the papers showed that most of the instruments, including the very complex ones, were made in the laboratories; this fact shows that highly skilled staffs and good facilities are available. Progress in radiation research is now to be expected only if physical and biophysical methods are widely used, and if varied radiation sources and experimental facilities are available.

2. RADIATION CHEMISTRY

Three special symposia and five sections dealt with radiation chemistry, on which more than 70 papers (i.e. some 20 per cent of all the papers) were given. The papers which were given at the symposia were on themes that the Organizing Committee had selected, while those which were given at the sections were on topics that the authors had selected.

Dainton gave a paper on radiation chemistry at the plenary session that was held on the day the congress opened. He noted that the subject had grown up rapidly, and outlined all its subdivisions; he then dealt in detail with one of the main topics, on which he had done much work (the radiation chemistry of aqueous solutions), and reviewed the current state of the subject. Much progress has been made in recent years; the primary processes related to radiolysis of water are now beginning to be dealt with quantitatively, instead of qualitatively as hitherto. The time scales of the successive radiation-induced reactions were reviewed; special attention was given to processes that remove free radicals, and to processes that compete with recombination when radical scavengers are added. The effects usually become important only at fairly high scavenger concentrations, depending on the scavenger properties. The spatial distribution of the radiolysis products from water was also dealt with; it was emphasized that the track density is related to the number of radicals which avoid combination. The results relate to deuterons and helium ions of various energies, and form a major contribution to the general theory of radiation effects in aqueous solutions; they are of some interest to radiobiologists also. Finally, some data were given on radiation effects in mixtures of the vapours of light and heavy water; they show that 12 molecules of water are involved per 100 eV absorbed, and not 3–4 molecules.

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Dainton dealt with current basic problems in the radiation chemistry of aqueous solutions, but did not mention Soviet work on some of the topics (the effects of acceptors on radical combination, and the role of excited molecules). The cause is probably that our publications do not enjoy a wide circulation and are inaccessible.

Smoluchowski (Carnegie Institute, Pittsburg) read his paper "The effects of radiations on solids" at the same plenary session. He reviewed the theory of such effects, and remarked that it had been developed for defects caused by collisions, but that it was less satisfactory for effects caused by ionizing radiations, and that it cast no light at all on the way defects change the properties of the solid. It was pointed out that γ-rays and fast electrons can cause defects, as can fast neutrons and heavy particles; in KCl crystals, for instance, Cl^- ions are doubly ionized to give Cl^+, and the Cl^+ ions readily enter the lattice. Electrons of energy over 50 MeV may enter the nucleus. In either case we get defects that alter the properties of the crystals. Some data were also given on other changes induced in solids by radiation (in the energy, resistivity, linear dimensions, diffusional behaviour, reduction and reaction rates, and catalytic properties), and it was shown that temperature affects the residual effects.

Symposium I. The roles of oxygen and of peroxides in radiation chemistry

Five papers were presented, which covered many aspects of processes that involve oxygen and peroxides in gases, liquids and solids.

(1) *Proofs that there are intermediate reaction products formed from oxygen in solids, liquids and gases.* (Giguere, Quebec, Canada.) This paper was a critical review of the published data; of the possible products such as oxygen atoms, excited molecules, OH radicals and HO_2 radicals, the two latter were dealt with in detail. It is considered proved that OH exists in the gas phase, but OH has not been detected in condensed phases. The HO_2 radical, which has often been invoked in chemical kinetics, has been detected for the gas phase in the mass spectrometer only; no evidence for its existence in solids or liquids has been found by any method.

(2) *The role of oxygen in gases.* (Noyes, Rochester, U.S.A.) The various radical reactions considered were those that occur in photolysed gaseous hydrocarbons, alkyl iodides, acetone, etc.; the effects of molecular oxygen on these reactions were also dealt with. The various excited states that the radicals might have were detailed in the interpretations which were given to the processes.

(3) *The roles of oxygen and of peroxides in aqueous solutions.* (Weiss, Newcastle, Great Britain.) Some new results were presented on the radiation chemistry of oxygenated aqueous solutions of complex organic compounds. Special proof was given that hydroperoxides exist under these conditions.

(4) *The roles of oxygen and of peroxides in organic liquids.* (Bakh, Moscow.) The processes dealt with were those in the oxidations of irradiated hydrocarbons; it was shown that the reactions may or may not be of chain type. The two types were illustrated by considering hydrocarbons and ethers, respectively. It was pointed out that a typical feature of radiation-induced reactions is that they are driven by the excess energies of the primary products.

(5) *The roles of oxygen and of peroxides in organic solids.* (Magat, Paris.) Radiation polymerization and the effects of radiation's acting on polymers in the presence of oxygen were dealt with. Chain reactions do not occur, but crosslinking (graft copolymers) occurs because hydroperoxides and peroxides form and decompose. Oxygen combines at the double bonds that radiation produces by splitting off molecular hydrogen.

The various oxidative processes that occur in the various media under different conditions could be compared at this symposium; it is clear that the processes have much in common. The time allotted to discussion was unfortunately short. It is clear that Soviet work on radiative oxidations occupies a substantial position.

Symposium II. The free radicals that are produced by radiations

Five papers were presented at this symposium on a topic of primary importance in radiation chemistry.

(1) *Possibilities and limitations of e.p.r. methods in radiation chemistry.* This paper was given by Livingston (Oak Ridge), who is one of the main workers on this subject in the U.S.A. He reviewed the experimental aspects of the method briefly, and then dealt with relative and absolute measurements of the free-radical concentrations that radiation produces in solids. He specially emphasized the need to use monocrystals instead of polycrystalline materials, since the effects are found to be orientation-dependent. He gave data showing that atomic hydrogen is produced when vitreous and crystalline perchloric and sulphuric acids are irradiated; he also gave data on

ethyl alcohol which showed that certain radical species are produced in low-temperature irradiations. In conclusion he pointed out that hasty deductions should not be made, since radicals if present did not always give signals, and a signal could not always be assigned to a definite radical.

(2) *Flash photolysis in radiation chemistry.* This paper was given by Porter (Great Britain), the originator of the method. The spectra produced by a very brief flash are studied; the method can always be used with free radicals in excited states. Many free radicals have been detected in this way. The method is much used in studies on primary photoreactions and on radical reaction kinetics.

(3)–(5) all dealt with the free radicals that radiation produces in biochemical and biological systems. The way the signals vanish has been used to study how oxygen, water, temperature and light intensity affect the signals that are produced by illuminating chloroplasts (Calvin, Berkeley, U.S.A.). Gordy (Durham, U.S.A.) discussed the influence of carbonyl groups on the oxygen effect. Zimmer (Heidelberg, West Germany) gave data on how oxygen affects the signals from irradiated barley seeds.

The papers all aroused much interest; e.p.r. methods, in spite of their limitations, form a very powerful tool for use in studies on the unstable products that form in many processes.

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Symposium III. Elementary physical processes

Lassettre (Columbus, Ohio) dealt mainly with the detailed comparison of the effects caused by photons and by electron impact. He showed that the two sets of effects were very similar in the cases of water vapour and of oxygen.

Hurst dealt with how negative ions are formed in oxygen, and gave data on the effective electron-capture cross-section of oxygen as a function of energy, and on electron capture in layers of oxygen and of nitrogen.

There were also five sectional meetings to deal with particular topics, in addition to the three symposia.

(1) *Energy transfer, role of excitation, γ-ray scattering calculations and effects of radiations on solid salts.* Flanders and Fricke's use of an electronic computer to solve for the diffusion kinetics of a solution with one acceptor attracted special attention.

(2) Calorimetric methods are being more widely used in dosimetry applied to radiation chemistry. Chemical dosimetry has also been further developed. Harlan and Hart (Argonne, U.S.A.) reported that the reduction of Ce(IV) could be used to measure doses up to 10^8 rad, and Hart described the dosimetry of recoil protons mixed with γ-rays.

(3) Various trends are evident in the radiation chemistry of organic systems.

Radiation synthesis. Sulphochlorination. Willard and his co-workers (Wisconsin, U.S.A.) have worked on hot-atom chemistry, and have compared the photolyses and radiolyses of alkyl iodides and bromides. They also gave papers on the radiosensitivity of polythene, damage and cross-linking in polystyrene, etc.

New data were given on the initial yields of hydrogen and of hydrogen peroxide that ultrasonics produce in water (Weissler, U.S.A.), in the section on aqueous solutions; yields of radicals in aqueous solutions and in organic liquids were also given.

Burton and others gave data which showed that hydrogen atoms are produced when organic liquids are irradiated.

The papers covered a great deal of ground, and related mainly to work done in the U.S.A. A recent development is that more concentrated aqueous solutions are being used, although these have long been studied in the U.S.S.R. Detailed studies were reported on the kinetics and mechanisms of radiation processes, including studies on the effects of ionization density, dose rate, etc., which were carried out on computers. Detailed comparisons of photolysis and radiolysis for organic systems were largely lacking, as in the U.S.S.R. also. There were only three papers on the effects of radiation on polymers and on polymerizations, for the reason that the topics are studied mainly at industrial research centres that are unwilling to reveal their results.

As a whole the papers were very interesting; in some cases it was clear that foreign workers were poorly acquainted with Soviet work.

3. RADIATION BIOCHEMISTRY SECTION

This topic was given much attention; Stocken (Great Britain) reviewed the subject generally at a plenary session, and there was also a symposium on DNA and chromosome structures; 35 papers were read at the four sectional meetings. The largest fraction of these papers dealt with the effects of radiation on nucleic acid and nucleotide metabolism; the next most important fraction dealt with enzyme systems. Some papers dealt with effects on aspects of *in vivo* metabolism such as steroid synthesis, oxidative phosphorylation, protein synthesis, etc. No essentially new facts or trends were revealed, in spite of the many papers that were presented.

Stocken's review at the plenary session contained some interesting generalizations from our present knowledge of disturbances in nucleic acid metabolism in irradiated cells. The close connections between disturbances in oxidative phosphorylation and depressed DNA and RNA synthesis were stressed. Phosphorylation in the nuclei is particularly important, since in radiosensitive tissues it falls to zero within an hour of their receiving 100 r doses.

Butler's paper at the above symposium was in the main a repetition of his interesting paper (*Radiation Research*, 1957) on the differences between the changes caused in DNA by ionizing radiations and by radiomimetic substances.

Latarjet's paper dealt with his work on the almost complete parallel between the effects of ionizing radiations and of bacterial transforming factor (which is pure DNA), in which he used succinic acid peroxide; this work again showed that peroxides play a major part in the indirect effects of radiation.

Maass's work with Yoshida sarcoma cells aroused great interest; it showed that glycolysis was suppressed while respiration continued, if the cells were irradiated *in vitro*. It was found that triose phosphate dehydrogenation was depressed because the diphosphopyridine nucleotide content was much lowered. The ATP content fell because there was less glycolysis, and so ^{14}C-alanine was incorporated into the proteins more slowly.

New details were also given on the lessened synthesis of DNA after irradiation (Potter, University of Michigan; Fliedner and Sherman, Brookhaven National Laboratory); tritium-labelled thymidine was the main compound used. Mice incorporated thymidine into nuclear DNA in the adenose epithelium at only 50 per cent

of the normal rate at 15 min after whole-body doses of 3 kr. Doses even of 5000 rad caused no sharp fall in the rate at which bone-marrow nuclei took up thymidine.

Some papers dealt with how nuclease was activated by irradiation (Tabachnik and Weiss, Philadelphia; Kurnick, California; Roth, Philadelphia Medical College). Tabachnik showed that RNAase in guinea-pig skin became much more soluble after the animals had been irradiated with ^{90}Sr β-rays. Roth showed that RNAase passed rapidly from the nuclei, mitochondria and microsomes into the supernatant from a homogenate of rat spleen at 16 hr after a whole-body dose of 700 r.

Mee (Cambridge, Great Britain) showed that the Michaelis constant of chymotrypsin was increased by irradiation, whereas heating caused inactivation without change in the constant; this showed that the mechanisms of inactivation by heat and by irradiation must be different. Fletcher (University of Rochester, U.S.A.) showed that enzymes were protected by being adsorbed on cellulose; this agrees with our view that energy migration plays a part in protection effects.

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Luzzio (Kentucky) has found that agglutins A and B (human) have different radiosensitivities; antibodies to bacteria also differ in radiosensitivity.

Several papers dealt with the disturbances in syntheses in irradiated animals; Gould and others (University of California, New Mexico, and Hammersmith Hospital London) dealt with the large increases in the rate of uptake of ^{14}C-acetate into liver and adrenol cholesterol in rats 24 and 48 hr after they had received whole-body irradiation. It was deduced that steroid metabolism is disturbed by irradiation; Soviet workers have found the same effect with micro-organisms.

Bernheim (North Carolina) dealt with antioxidant inactivation in the walls of the intestine; the result was that peroxides formed spontaneously after irradiations. No peroxides were detected in other organs. The data agree with some results from the U.S.S.R.; they do not show that prolonged branched-chain oxidations occur in the lipids.

Several papers, and Gray's review, dealt with the oxygen effect. A new feature was the finding that the inert gases exert very different protective effects. Gray (and in more detail Ebert) showed that the pressures needed to reduce the effects of 0·2 atm of oxygen to equal extents were 55 atm of helium or hydrogen, 12·5 atm of nitrogen, 2 atm of argon or krypton and 1·1 atm of xenon. The effects with the inert gases correlate with the latter's partitions between the aqueous and lipid phases, which may mean that the gases are sorbed differently by lipid centres on the surfaces of the cells.

Kuzin's paper dealt with the effects of ionizing radiations on *in vivo* sorption by tissues and cells, and was presented at the radiation physiology section. Butler (Great Britain), Brues (Argonne Laboratory, U.S.A.) and Ebert (Great Britain) participated in the discussion on this paper.

4. CELL RADIOBIOLOGY SECTION

This topic was dealt with in Gray's paper at the second plenary session; there was also a special symposium on the various radiobiological effects to be found in one species of cell (9 papers were read), and there were five sessions of the Cellular Radiobiology section, to which more than 50 papers were read. The topic was also dealt with in other sections (biochemical changes after *in vivo* irradiation, radiation

biochemistry, effects on nucleic acid metabolism, etc.), so the problem was discussed from all sides. This emphasis on the cellular level is quite justified, since the first changes in irradiated living organisms occur in the cells of radiosensitive organs and tissues. The trend in cytology that is most important to radiobiology, and had its appropriate place in the work of the congress, is the development of new and very convenient objects, such as animal and plant tissue cultures kept under precisely controlled conditions (particularly as regards synchronous division), algal cultures and yeast and bacterial cultures that show synchronous division. Human-cell cultures, including those of tumour cells, are of special importance to radiobiology.

New methods have also been developed for use in cell radiobiology, such as partial-cell irradiation from point-focus sources (radiation probes), which has been developed to a fine art in Zirkle's laboratory. Methods have also been developed of studying metabolism on cell structures using labelled purines and pyrimidines. Autoradiography has been used to locate and measure the activities. Tritium-labelled metabolites have been used to effect local irradiation and to give radioautographs of structures. Much attention was given at the congress to special irradiation conditions to be used with carefully controlled temperatures, chemical compositions of media, etc.

The more important results that were presented can be grouped under several headings. Comparisons of nuclear and cytoplasmic radiosensitivities were given much attention. Selective and local irradiation methods were used with nuclei and with parts of cells free from nuclei; in several cases it was thus shown that the nuclei and the separate chromosomes were highly radiosensitive. Cell reactions that depend on irradiation applied to the cytoplasm were also detected (C. L. Smith, Great Britain; Ulrich, Switzerland; Zirkle *et al.*, U.S.A.; etc.). Damage caused to cell nuclei by ultra-violet light can be reduced by photoreactivation, whereas damage to the cytoplasm cannot (von Borstel and Rekemeyer, U.S.A.). Proton and ultra-violet microbeams were used to show that chromosome sticking depends both on the direct and on the indirect effects of radiation on the chromosomes (Zirkle).

The oxygen effect also received much attention; various explanations were proposed. Ebert and others conclude from their studies on the effects of inert gases that oxygen and the inert gases compete for sensitive lipid structures in the cells. Alper explains the oxygen effect in terms of irreversible damage to excited molecules which can be reactivated if oxygen is absent. These views accord with those that have been developed in the U.S.S.R.

Stress was laid on the importance of depressed DNA synthesis to the reduced mitotic activity of irradiated cells. It was shown that division is more sensitive to irradiation than is DNA synthesis, and that the two processes are therefore not directly coupled. Shaw (U.S.A.) showed that mitosis suppression had different mechanisms at low and high doses.

Forssberg (Sweden) analysed the time delay in division in *Phycomyces* cells given localized doses as low as 0·01 to 1 r. This effect he assigned to the failure to form energy-rich compounds.

Several good papers dealt with polyploidy in relation to radiosensitivity; the two were shown to be directly related in rice and chrysanthemums, with the effect particularly apparent in natural polyploids.

Reactivation and recovery at the cellular level were also dealt with. Latarjet (France) showed that slightly damaged cells adjacent to ones inactivated by irradiation could not assist the latter to recover, although this effect had originally been reported from Delaporte for bacteria. Alper and others analysed how irradiated micro-organisms recovered when they were cultured under suboptimal conditions. They explained this interesting effect in terms of the loss of an unbalanced equilibrium in the synthetic processes when the cells were cultured in conditions unfavourable to growth and division.

Pollard (U.S.A.) gave a masterly analysis of the radiation damage to sulphur metabolism in bacteria. Labelled sulphate and methionine were used to show that the uptake of sulphate into irradiated cells was depressed by nine times as much as was the uptake of methionine. His results from deuteron bombardment of the cells were such as to indicate a very simple mechanism for the effects of radiation on sulphur metabolism.

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There were several papers on the causes of the differences in radiosensitivity that are found with different cells. Goldfeder's paper "A correlation of cellular structure and radiosensitivity" was of special interest. Cells from two tumours in the same organism were used; the more radiosensitive tumour showed more energetic anaerobic glycolysis and oxidative phosphorylation, and its cells contained more numerous and better organized mitochondria.

Meisel' (U.S.S.R.) presented results from the Institute of Biophysics and from the Central X-ray Research Institute on the early structural changes and states caused by radiation in cells from radiosensitive organs; these results were obtained by fluorescence microscopy. It was shown that the damage could be detected in this way almost as soon as the irradiation had finished. The paper aroused much interest.

5. THE EFFECTS OF RADIATION ON VERTEBRATES

The main attention was here given to the effects of different forms of radiation (X-rays of various energies, γ-rays and neutrons applied locally or generally). The biological effectiveness of the various forms was measured. Loken, Beisang, Mosser and Marvin compared the effects of 220 kV X-rays and of ^{60}C γ-rays on hens' eggs. Upton, Sproul and Randolph used single-shot and fractionated doses of neutrons, X-rays and γ-rays with mice. Tochilin, Shumway, Swift and Taket compared 250 kV X-rays with fast neutrons applied locally to rats.

Sinclair, as also Kereiakes and Krebs, used yeasts, mice, rats and hens' eggs to measure LD_{50} and minimum lethal doses. It was found that neutrons gave the greatest effect, X-rays a less one, and γ-rays the least of all, if the conditions were comparable.

Relatively few papers were devoted to the pathogenesis of radiation sickness; much more has been done on this topic in the U.S.S.R.

Mole outlined the effects of radiation on mammals, and showed that to assess the radiosensitivity was much more difficult in higher organisms than in lower ones. He pointed out several special features of radiation after-effects in mammals. He made a serious error in placing the nervous system last in radiosensitivity. He also dealt with dose rate and time effects and, comparing single doses with chronic irradiation, directed attention to the remote effects.

Several papers dealt with the reactions to radiation of the endocrine organs, of the cardio-vascular system and of the digestive tract. It was remarkable that there was not even one paper on the role of infection in radiation pathology.

6. THE EFFECTS OF INCORPORATED RADIOACTIVE SUBSTANCES

The papers in this section were presented at two sessions. Graul dealt with biological effects caused by materials accumulating in tissues. Dockum, Case, Vogt, and Stover dealt with the effects of plutonium, and gave the distributions of ^{239}Pu in tissues, the amounts that accumulate in bone, and the use of autoradiography for detecting plutonium in human tissues. Johnston, Asling, Stover, Nelson and others dealt with the deposition and behaviour of ^{90}Sr, together with ^{45}Ca. Many data were given on carcinogenesis by incorporated isotopes. Watanabe dealt with the pathogenesis of osteogenic sarcomata induced in mice with ^{89}Sr; Casarett, Conley and others reported similar work on rats. Mortality and incidence statistics were given as functions of dose of strontium, and the mechanisms whereby radiations produce osteosarcomata were discussed.

Bustad, George, Marks and others have given daily doses of radio-iodine that varied from 0·005 to 1800 μC, and have found a gradual wave-like fall in the thyroid gland's activity. In some cases tumours developed in the gland. Durbin, Asling and others have studied the effects of ^{223}Ra on bone-marrow cells, using the uptake of radio-iron into erythrocytes. This uptake they found to decrease gradually; the number of bone-marrow cells also fell. Bair, Temple, Willard and others have caused mice to inhale dusts containing ^{131}I, ^{90}Sr, ^{239}Pu and ^{106}Ru in small amounts for periods of 75 days; they have studied how these materials accumulate in the lungs and in other tissues, and what the changes are that the radiations cause. The materials were found to be in the lungs during the first weeks, and to be transferred to other sites subsequently (plutonium to the muscles, strontium to the bones, etc.). Carcinomas, fibrosarcomas and lymphosarcomas developed in the lungs.

Marinelli attempted to correlate the radium content of drinking water with the frequency of bone tumours; although the two sets of data showed parallel trends, the difference between the numerical coefficients was small (1·78–2·17). The data were also too few, and further studies are needed.

Muth, Rajewsky, Hantke and Aurand have studied the ^{226}Ra contents of food products, drinking water and of human tissues; the skeleton showed the most radium (a fact already long known). The placenta and foetal tissues showed the least radium. The bones of growing organisms take up more radium. Milk and potatoes show the largest radium contents.

7. REMOTE AFTER-EFFECTS OF RADIATION

Internal and external radiation sources were used in the work reported at these sessions. Mewissen showed that, in mice of the 57 Black strain, 10 per cent developed tumours after doses of 283 r and 90 per cent after doses of 800 r. The numbers that developed tumours were reduced slightly if ^{60}Co was used and antibiotics were given.

Faber reported on the development of leukaemia in 13 radiotherapy patients; he found that the time from treatment to onset of leukaemia was less (10–40 months)

if the leukaemia was of the acute form than what it was (20–140 months) if the disease was of the chronic form.

Murray and Hempelmann have observed leukaemia in 20 patients who had been treated by radiotherapy while they were children.

Cater, Lisco and Baserga have studied how tumours develop in the bones and soft tissues of rats that had been irradiated with ^{192}Ir γ-rays. Osteosarcoma, skin and mammary cancer, and fibrosarcoma were found. Hormone treatments did not change the incidence of the tumours in any way.

Bennett and others have studied how dose fractionation affects late complications and radiation carcinogenesis in rats. Complications were found more frequently with fractionated doses.

Papers were also given on how radiation reduces life-spans and induces cataracts.

Conard's paper dealt with the complications which were found in inhabitants of the Marshall Islands and which were caused by the fall-out from the hydrogen bomb exploded at Bikini in March 1954. Various forms of skin damage were observed, and permanent loss of hair, partial baldness, atrophy and depigmentation in the skin, and teleangioactases. Slighter changes were found in the peripheral blood; the thrombocyte count was slightly increased, and the neutrophil count slightly decreased.

Several papers also dealt with the effects of radiation on experimentally induced tumours. Straube, Patt and York found that more Erlich ascites tumours arose if cyanides and oxygen were given during the irradiations.

Mori tested several isotopes on the cells from an experimental sarcoma. Stroud, Brues and Richard used various forms of radiation and varied the irradiation conditions in their studies on a malignant process.

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Publishers' Notice to Subscribers of Soviet Biomedical Journals published by the Pergamon Press about an increase in frequency, number of pages published and subscription price.

Due to the increased number of good manuscripts being submitted for publication to *Biophysics* the Soviet Editors and Publishers have decided to increase the frequency of publication from bi-monthly to monthly. It will therefore be necessary for subscribers to pay an additional £10 6s. ($30) for the 1959 volume and the subscription price for the 1960 volume will now be £21 ($60).

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